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Nervous Structure of the Spinal Cord of the Young Larval Brook-Lamprey

BY

H. P. WHITING, D.S.C., PH.D.

(Department of Zoology, University of Cambridge)

With two Plates

IT is intended to describe here the spinal cord of recently hatched ammocoetes of *Lampetra planeri* (Bloch). Particular attention will be given to those neurons which may be involved in the somatic sensori-motor arc. It is hoped to base upon this description a subsequent account of the embryo and young ammocoete, which will deal with the successive stages of nervous structure and their relation to the successive patterns of behaviour.

The neurology of the ammocoete, or pride, should be of particular interest, because great importance attaches to all aspects of the morphology of this animal in discussions of the phylogeny of vertebrates. For although the gnathostome vertebrates are not regarded as derived from some palaeozoic ammocoete, the morphology of the ammocoete certainly resembles that of the prototype from which higher vertebrates may be said to have evolved. This point of view is supported by the accounts of the general morphology of the nervous system of the young stages, and is borne out by the detailed neurology of the brain in the older stages and in the adults.

With very minor exceptions, the description of the nervous system of the younger stages has hitherto been made without the use of metallic impregnation or any other method of staining nervous tissue specifically, except in animals of above 4 cm. in length, that is in animals more than 1 year old (cf. Hardisty, 1944). But knowledge of the very youngest stages is urgently required; for the nervous organization of these could be compared with that found in amphibian embryos: and it is by a study of that organization in the embryos of the urodele that Coghill (1914, 1929, &c.) and Youngstrom (1940) have erected a far-reaching system of correlation between the successive structural patterns of the neurons during development and the stages of development of behaviour.

It is important to know how far Coghill's and Youngstrom's conclusions are applicable to other classes of vertebrates. Similar investigations to those of Coghill have been performed very effectively upon placentals (Barron, 1941). Upon fishes similar lines of research have been less successful. Descriptions of nervous structure almost all deal with a late period of development, when swimming movements are already an integral part of behaviour; when the most critical period in the relation between structure and function is already

past history. The few accounts at appropriately early stages of development deal chiefly with sensory or motor neurons which have peripheral processes. This applies to that of Neal (1914) on *Squalus* and to Harrison's classical account of the Rohon-Beard cells of *Salmo* (1901).

On the other hand, good descriptions of the early stages of behaviour are also rare. The few that exist, such as that of Wintrebert (1921) on *Scyliorhinus*, are difficult to assess in the absence of knowledge of the contemporary nervous structure.

In fishes we lack an adequate picture of the sensori-motor arc in the nervous system during the period when locomotor movements or those of the pectoral fins and visceral arches are being developed. In cyclostomes the position is even less satisfactory. For this group there is no description of the relation between the neurons of the spinal cord in the young. Yet such a description in vertebrates so near the prototype is clearly fundamental.

The present paper attacks this problem in the Petromyzontia. The detail of the nervous structure will be described at the period in which locomotion by swimming movements has just been achieved. The animal is then at a stage roughly comparable with the stages described in *Amblystoma* by Youngstrom.

Previous Work on the Petromyzontid Nervous System

The extensive literature on the neurology of lampreys has been well summarized in Kappers-Huber-Crosby (1935). The descriptions of the brain are relevant to a study of the spinal cord in that they show that the brain, at least, appears to be organized in a very primitive fashion. The simple form of the brain in the young ammocoete can be seen from von Kupffer (1906), figs. 47 to 57. The primitive and surprisingly unspecialized neural architecture and tracts of the adult brain are clearly described in Johnston (1902). Later work, such as that of Barnard (1936), Woodburne (1936), and Pearson (1936), has confirmed his conclusions.

Work on the neurology of the brain has been mainly devoted to adult or advanced ammocoete preparations. For instance, Tretjakoff (1909*b*) gave an extensive account of the brain of ammocoetes of length 10 to 18 centimetres. In passing, it is important to note that fig. 43, Tretjakoff (1909*b*), is reproduced in Kappers-Huber-Crosby (1935), fig. 316, where it is wrongly described as part of the brain of an 'Embryo'. But Larsell (1947) has recently described some parts of the brain in younger ammocoetes: the smallest of his silver-impregnated specimens was 42 mm. in length.

In contrast to the brain the spinal cord of the older ammocoete and adult lamprey is not of the form to be expected in a prototype, and the function and homology of the constituent neurons are difficult to understand. Kolmer (1905) and Tretjakoff (1909*a*) have been the chief contributors on the subject. Their conclusions are discussed by Kappers-Huber-Crosby, but the original figures should be consulted. The unusual characters of the spinal cord may be summarized as follows. The cord is so broad and flat as to be almost

ribbon-like; the positions of dorsal, lateral, and ventral funiculi are therefore abnormal. Dendrites of motor neurons extend across their own half of the spinal cord, and may extend to the contralateral side, either above or below the neural canal: the peripheral motor fibre is derived from a collateral of a longitudinal fibre. The chief form of intercalary cell is very large: its dendrites extend into the contralateral dorsal funiculus, while its axon runs longitudinally and then crosses into the contralateral ventral funiculus. Giant fibres descend in the ventral and lateral part of the cord from the giant cells of Müller in the midbrain and hindbrain; these are believed (e.g. by Stefanelli and Camposano, 1946) to be co-ordinating fibres comparable to the Mauthner fibres of teleosts and urodeles, but they occupy a considerable part of a transverse section of the cord. In a dorso-medial position in the grey matter are found some very large cells, termed *Hinterzellen*, whose axons run in the dorsal funiculus. There are normal dorsal ganglia, but the dorsal and ventral roots do not unite in the usual manner.

Two points in the description of the cord of adults and the older larvae require special attention. Firstly, in a Viennese journal, Sigmund Freud (1877) described somatic-sensory fibres which he claimed and figured as originating from the Hinterzellen, i.e. from cells of the dorsal horn. Now in the larvae of fishes and amphibia there are large neurons within or just above the dorsal part of the spinal cord which have the relations of somatic-sensory dorsal ganglion-cells; these neurons are termed Rohon-Beard cells. If Freud's figures were correct, there would be a strong presumption that the Hinterzellen are homologous with Rohon-Beard cells, especially since Rohon-Beard cells are persistent in many adult teleosts. This relation between the Hinterzellen and the dorsal root was subsequently denied by Tretjakoff (1909*a*). But it was again described by Beccari (1909), who supported his case with some very clear figures.

Secondly, there is a well-developed Commissura Infima Halleri at the anterior limit of the cord. This commissure was described in the lamprey by Johnston (1910), but his paper was not mentioned by Kappers-Huber-Crosby. The importance of the functions of the Commissure of Haller are emphasized by Herrick (1944), and it can be seen, from a comparison of Johnston's account of its structure with the description by Herrick (1908) of its relations and its functions in fishes, that in lampreys this commissure is probably concerned with a correlation between the left and the right spinal somatic-sensory components, and also between these and the cranial nerves.

Turning to the embryo and the young pride, we find three descriptions of the large dorsal cells in the spinal cord; the first two of these have been overlooked in Kappers-Huber-Crosby's account. Kupffer (1894) showed a 'Rohon'sche' cell, i.e. a Rohon-Beard cell, lying just dorsal to the cord in a 3-mm. embryo. Studnicka (1895) figured Hinterzellen in *L. planeri* of lengths between 3 mm. and 30 mm. In his younger stages, the Hinterzellen now lie just below the dorsal surface of the cord; in the older stages, they have sunk still farther into a position ventral to the dorsal sectors of the white matter.

The axon runs longitudinally in the white matter; the peripheral process appeared to him to be independent of the dorsal root, and free from metameric organization. Studnicka concluded that the Hinterzellen were probably motor in function. Beccari (1909), using a silver impregnation, described the same cells in prides of 20 mm. His account confirms that of Studnicka, except that he found that the peripheral fibre left the cord as part of the dorsal root. It seems clear from the three papers that the large dorsal cells are true Rohon-Beard neurons, for they have very large cell-bodies which begin in a very dorsal position in the cord, an axon running longitudinally in the dorsal white matter, and a peripheral fibre which is presumably sensory, since in the older stages it accompanies the other dorsal-root fibres.

The only other type of neuron described in the spinal cord of these stages of *Lampetra* is the motor neuron. Kupffer (1890) described the first motor axons running from the spinal cord to the myotomes in 3-mm. embryos (his fig. 81).

The peripheral nerves of the trunk in the newly hatched pride have been described by Sagemehl (1882), Shipley (1887), Dohrn (1888), and von Kupffer (1890). Von Kupffer states that the ventral spinal nerve, on leaving the cord, passes ventrally between the notochord and the inner face of the myotome. He describes the dorsal nerve as emerging from the cord and dividing into a lateral and a median branch: the median branch turns ventrally, to pass between notochord and myotome, while the lateral branch passes outward across the upper surface of the myotome. The dorsal ganglion-cells are figured as lying on the course of the median branch of the dorsal nerve, e.g. his figs. 81 and 82.

The relation between the dorsal ganglion and the dorsal nerve was a matter of dispute between these authors. But it would have been difficult to establish the facts with certainty by the aid of the stains they were using: von Kupffer, for instance, used borax-carmine. Certainly, the cells of the dorsal ganglia of the spinal cord are still in a very early stage of development (see Shipley). Methylene blue preparations of peripheral nerves in much older ammocoetes have been described by Tretjakoff (1929). His fig. 25 shows a dorsal spinal nerve dividing into four branches. Two of these branches correspond to the median and the lateral branches of von Kupffer's account: but the other two run, one dorsally and the other dorso-laterally, quite clear of the myotome to reach the skin. His fig. 26 shows a motor neuron.

The position reached by previous work is, then, that in the newly hatched larva the sensory and motor somatic nerves have reached their end-organs; that the sensory fibres are at least partly derived from Rohon-Beard neurons or other intramedullary neurons very like them; and that the dorsal ganglion-cells on the other hand are at an early, probably only neuroblastic, stage of development. In short, the peripheral relations of the nervous system in the trunk have already been considerably elucidated. On the other hand, very little is known about the neurology of the spinal cord. Some of the central

relations of the sensory neurons might be deduced from Studnicka's work and Beccari's work on older animals. But nothing is known about internuncial neurons and the correlating mechanism; nothing is known about the development of descending fibres from the brain which might by this stage be co-ordinating the motor tracts; nor have either the dendrites or the longitudinal processes of the motor neurons been described.

Methods

Two methods of staining neurons specifically were used, namely, vital staining with methylene blue and 'silver on the slide'.

Methylene blue does not appear to have been used previously on the nervous system of vertebrate embryos. It has many advantages.

1. Much younger material may be used than will adequately take other nerve-specific stains, or impregnations.
2. Total preparations may be made of the more transparent embryos such as *Salmo*. These show much that would be difficult to perceive or reconstruct from sectioned material. This is especially true of nervous structures which are segmentally repeated.
3. The neurons which have taken the stain may be examined either in the living animal or in permanent preparations.
4. Methylene blue and Golgi preparations have many similar characteristics, as Polyak (1941) has pointed out. The sections may be relatively thick, 30μ to 60μ. Only a limited number of neurons are shown up, but these take the stain throughout, in nucleus, cell-body, axon, and dendrites. Methylene blue and Golgi sections are therefore relatively simple to interpret.

In the case of lamprey material, the presence of opaque white food-material in the cells of the younger embryos makes it difficult to observe the central nervous system in the living animal by means of methylene blue.

Silver was used to complete the picture given by the methylene blue. Since silver impregnates all neurons a more quantitative account is possible. Its use also ensures that no entire category of neuron shall be omitted from any reconstruction, for it is exceptional for methylene blue to colour some categories, e.g. the Müller and Mauthner giant fibres.

A silver method where reduction takes place on the slide is more easily controlled than one of the Ramón y Cajal methods, and is therefore particularly suited to very young material, for which adjustments in any technique are usually necessary.

As a control method embryos fixed in Susa were stained by a variety of standard techniques. The most effective was Mallory's phosphotungstic acid haematoxylin, which gave good differentiation and also picked out the neuroglial cells.

Methylene blue. The stain may be applied either as a coloured solution, or as a colourless solution of Rongalit-methylene blue. The former appears not

to penetrate so well. The results with Rongalit-methylene blue are, on the other hand, less predictable, so that it is less easy to determine beforehand what nervous elements shall take the stain. In each case, the quality of the methylene blue is important. In this work, Grübler's methylene blue for 'Vitalfärbung, nach Ehrlich' was used.

The coloured solution was applied to embryos such as trout and lamprey at about 10° C. at dilutions of about 1:1000. The embryos were completely immersed. The first staining, apart from any tissue which might have been cut or damaged, usually occurred after 2–5 hours. Usually nerve-cells, especially those with long peripheral fibres, were the first to appear blue. Coloration is intense, so that there is usually excellent contrast between stained and unstained elements. Where there is a large yolk-sac, as in the trout, it is advisable to cut away the ventral part so that the dye can reach the coelom directly.

Rongalit-methylene blue was applied by the method used in this laboratory by Dr. C. F. A. Pantin, F.R.S., and by Dr. J. E. Smith upon invertebrate material. Their suggestions concerning its use were very helpful. The method is described by Smith (1946).

Whether using methylene blue or its colourless leucobase, the resulting preparations may be made permanent. The procedure has been developed from that of Cole (1936). Only on neurons which are still intensely stained should fixation be attempted, because some time elapses before the fixative affects the dye. *Stages 1–6 are carried out at between 0° and 3° C.*

1. The embryos are fixed for 2 hours in 8 per cent. ammonium molybdate. If it is necessary to fix overnight, difficult material should be given a preliminary fixation in saturated ammonium picrate *of 5 minutes' duration.*
2. Wash in water for 1 minute.
3. Transfer to the following *n*-butyl alcohol mixtures in the Lang (1937) series for 30–40 minutes each: 30, 57, 82, 91, 97 per cent. total alcohol.
4. Leave in absolute *n*-butyl alcohol for 2–12 hours.
5. Transfer to equal parts of methyl benzoate and *n*-butyl alcohol, then to pure methyl benzoate, for 1 hour in each.
6. Transfer to a mixture of methyl benzoate and liquid paraffin for 1 hour.
7. Transfer to ice-cold liquid paraffin. The embryos may now be allowed to come to room temperature. They may be left in liquid paraffin indefinitely, before they are examined as total preparations, or embedded in wax and sectioned.

The purpose of this procedure is to avoid the leaching effect of water or ethyl alcohol so far as possible. The following additional points may be helpful. (i) Longer immersion in ammonium picrate, as in the technique of Bethe (1898), causes the nerves to appear green and the background yellow. (ii) Diethylene dioxide has been used for dehydrating methylene blue material, but has not been found so satisfactory as the *n*-butyl alcohol technique. (iii) If the specimens are not to be sectioned, they may be brought from

methyl benzoate through a mixture with xylene and Canada balsam into balsam. Balsam preparations of *Salmo* embryos with stained neurons have kept excellently for the period of the war.

Silver. Embryos, narcotized in weak urethane, are immersed for 2 days in the alcohol-chloral hydrate fixative used by Nonidez (1939). They are then dehydrated with 57, 82, 91, 97 per cent., and absolute alcohols. These alcohols are made up according to the procedure of Lang (1937), which utilizes the properties of *n*-butyl alcohol, and is less damaging to delicate tissue than ethyl alcohol dehydration. The specimens are brought slowly through methyl benzoate, then liquid paraffin, and then imbedded in paraffin wax.

The methods of Nonidez and Lang enable considerably better impregnation to be achieved than is attained on similar material by more orthodox procedure.

The subsequent treatment followed the technique of Holmes (1943), which is one of the best of the many variations of the method of Bodian (1936). On the ammocoete material, good results were obtained using a 1 : 30,000 solution of silver nitrate in distilled water, buffered at pH 8·3 at 37° C. for 2–3 days.

Bayer's German pre-war Protargol was also used in place of silver nitrate, and gave good results. This form of Protargol is now difficult to obtain. The Protargol should either be used with addition of metallic copper, as in Bodian's method, or should be buffered to about pH 8·0 with the borax-boric acid buffer used in the Holmes method.

Professor J. E. Harris, Department of Zoology, Bristol University, has given me much important advice upon the silver procedure.

Counterstaining, e.g. with Orange G, is sometimes an advantage.

Material

The prides used in this study were between 7 and 10 mm. in length. They correspond to the developmental stage VIII of Hatta (1896), in which the stomodaeum has just opened. The general morphology of the central nervous system of these specimens closely resembles that figured by von Kupffer (1906) for the 6-mm. animal: no appreciable change toward the form described by him for the 20-mm. ammocoete has been observed.

The material was obtained from two sources:

(i) Prides of *Lampetra planeri* from a tributary of the Lymington river, in the New Forest. I am greatly indebted to Mr. A. R. Hockley, Lecturer in Zoology, University of Southampton, for the living and the fixed material from this source, and also for demonstrating to me his methods of collection.

Two batches from the New Forest were used for the silver preparations. Batch 'A', average length 7 mm., was fixed 10 days after fertilization. Batch 'B', average length 8 mm., was fixed 16 days after fertilization.

(ii) *Lampetra* prides, of 8–10 mm. in length, received alive from Professor J. E. Harris. These were used for methylene blue preparations.

Sagittal and transverse serial sections were prepared of both batches for the silver method: horizontal sections were prepared only of the 'B' batch.

Impregnation and differentiation of nervous tissue both in brain and spinal cord and peripherally was satisfactory in all sections.

The Nervous System of the Trunk Region of Young Ammocoetes

Sensory System

The chief sensory system at this stage consists of neurons having the form and relations of Rohon-Beard cells: they will be described under that name.

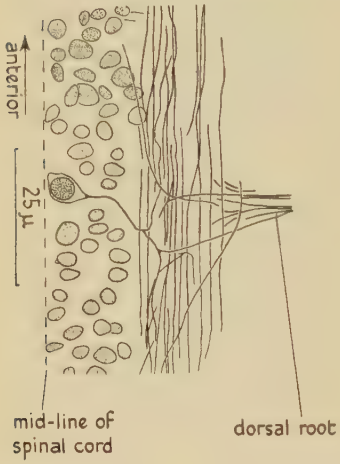


Fig. 1

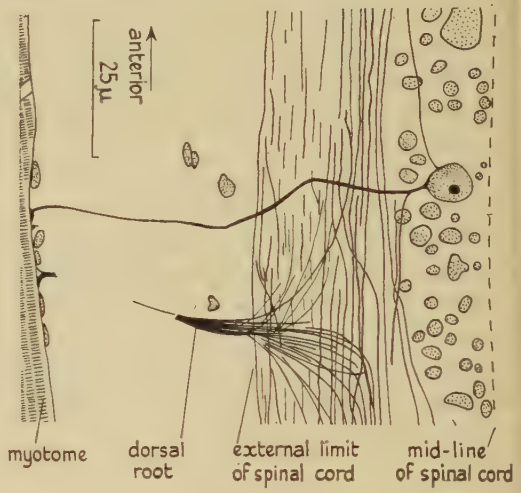


Fig. 2

Text-fig. 1. Horizontal section, 16μ, silver impregnation; from anterior trunk region of 16-day *Lampetra*.

Text-fig. 2. As Text-fig. 1, but nearer the head. These and the succeeding figures were drawn by means of a camera lucida, using $\times 45$ and $\times 95$ objectives.

Rohon-Beard neurons. The cell-body lies in the dorsal grey matter, with its median surface close to the neural canal. It is rounded and large, its diameter being about 6μ, or about twice that of any other type of neuron in the dorsal part of the spinal cord. Each neuron has three large processes: two turn outward into the white matter, where one fibre proceeds towards the head, and the other caudally: the third process runs outward, leaves the cord at the dorso-lateral edge, and runs to the somatic muscle or to the skin (Text-fig. 1).

At anterior levels the peripheral fibre usually lies in about the same transverse plane as the cell-body, and the cell-body is unipolar, as in Text-fig. 1. At posterior trunk levels the peripheral fibre leaves the cord some distance caudal to the cell-body, and is derived, as a collateral, from the descending fibre. The descending and ascending fibres originate from the cell-body separately, so that at posterior trunk levels the cell-bodies are seen in horizontal section to be consistently bipolar.

The longitudinal fibres can be traced for a distance equal to several somites: they give off slender branches, which appear to be quite short. The dorsal

white matter seems at this stage to be largely made up of the longitudinal Rohon-Beard fibres. The ascending fibres of Rohon-Beard cells lying near the head appear to run into the Descending Trigeminal tract of the medulla. The peripheral fibre normally runs out in the dorsal root, of which it is the largest, and at this stage the most numerous, component. Such a typical condition is seen in Text-fig. 1. But frequently one fibre runs out separately from those in the dorsal root. When such a separation is in the horizontal plane the outlying fibre has a quite normal course after reaching the endochondral layer (Text-fig. 2).

The cell-bodies lie in two rows, one on each side of the midline, for the whole length of the trunk. They are at various dorso-ventral levels: some project above the general outline of the grey matter, while others are as far ventral in it as the area which would in gnathostomes contain the viscerosensory component. No difference in the peripheral destination of the more dorsal and the more ventral cells could be observed. Presumably, in older ammocoetes, the cells of the two rows would settle into a single dorso-ventral level.

The dorsal root passes through the endochondral tissue at the intersegmental position, opposite the myocomma. On the outer side of the endochondral layer the root traverses the area of the dorsal ganglion, which lies at a dorso-ventral level midway between dorsal and ventral roots. Some Rohon-Beard fibres of the dorsal root pass beside or even between the cells of the dorsal ganglion without being in any way connected with them. This independence could be seen in transverse and parasagittal silver sections; it is probably true of all Rohon-Beard fibres, although the dorsal ganglia are so compact that it was not possible to be certain of the independence of every one of the many Rohon-Beard fibres examined (Text-fig. 5*a*). In methylene blue preparations no stained dorsal ganglion-cells have been found: stained Rohon-Beard fibres are seen to pass from the endochondral layer directly into the interface between successive myotomes.

Between the myotomes the Rohon-Beard fibres spread out and branch in lateral or ventral directions. The larger branches reach the skin. Small branches ending as proprioceptors on the ends of the myofibrils remain in the myosepta. No instance was found of branches penetrating in a longitudinal direction to end in parallel with the myofibrils (Text-fig. 3).

The Rohon-Beard fibres to the ventral part of the myotome and to the skin of the ventral surface do not penetrate between myotomes until they have passed well below the notochord: for this reason a parasagittal section just lateral to the notochord shows both the motor nerve and the ventrally directed Rohon-Beard fibres. The motor nerves lie in a mid-segmental and the Rohon-Beard fibres in an intersegmental position in remarkably consistent fashion through the whole length of the trunk.

On reaching the skin the Rohon-Beard fibres divide further; some subdivisions reach the dermis and may penetrate the basement membrane of the

epidermis (Text-fig. 4). Others continue down the outer side of the intersomite, where, by meeting other similar branches, they become an appreciable aggregate of fibres.

Just as some Rohon-Beard fibres run out separately from the dorsal root, slightly caudal or cephalad to it, so others proceed outward independently and dorsally to the dorsal root. Most of these fibres reach the dorsal tip of the myotome, where they cross the muscle in an intersegmental position, or pass round the top of the myotome to reach the skin. Since such fibres run

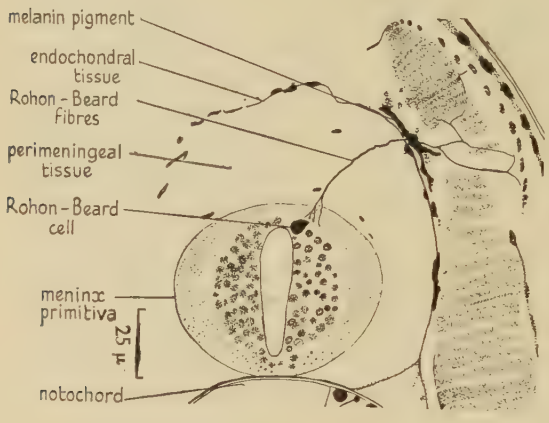


Fig. 3

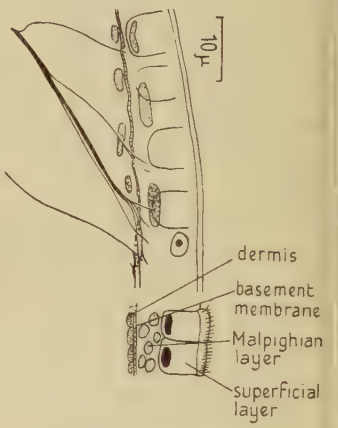


Fig. 4

Text-fig. 3. Transverse section, 16 μ, from a 10-day *Lampetra*; silver method. Shows the peripheral course of some Rohon-Beard fibres.

Text-fig. 4. Transverse section, 16 μ, from a 10-day *Lampetra*; silver method. Exteroceptive terminations of Rohon-Beard fibres. (Below, sketch showing appearance of the skin with standard histological methods.)

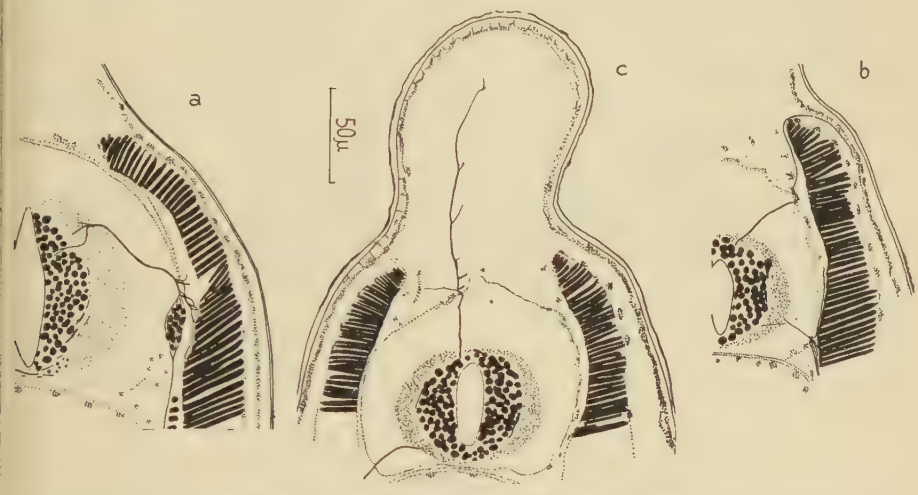
singly, and since they are clear of the dorsal ganglion on the whole of their course, they are easy to examine (Text-fig. 5*b*). Large branches of these fibres reach the skin, but the smaller branches turn back to end on a myofibril. Such Rohon-Beard fibres correspond closely to those described by Coghill in *Amblystoma*.

Others of the separate, latero-dorsally directed Rohon-Beard fibres do not reach the myotome at all, but pass from the dorsal surface of the spinal cord through the endochondral layer at only a slight angle to the vertical, so that they reach the skin of the dorsal surface. This third type of Rohon-Beard cell has been noted previously by Studnicka, Tafel III, fig. 11. He regarded the type as aberrant: but it occurs in small numbers at all trunk levels, and is evidently the only sensory neuron which innervates the dorsal areas of the skin in the young pride (Text-fig. 5*c*).

The Rohon-Beard fibres which proceed independently of the dorsal root are confined, like those in the dorsal root, to an intersegmental position.

When they occur at an intersegment in pairs they are arranged symmetrically about the midline. They are probably the 'pathfinders', the earliest sensory neurons to have developed at that intersegment, and neither of the independent types should be regarded as aberrant.

Dorsal Ganglion-cells. Some of the cells of the dorsal ganglia are round or pear-shaped neuroblasts: others are bipolar neurons which have slender peripheral and central processes. Neither form of process could be traced to its



TEXT-FIG. 5. *a*. 12 μ, silver method, 16-day *Lampetra*; Rohon-Beard fibres in the dorsal root. *b*. 16 μ, silver method, 10-day *Lampetra*; Coghill's type of Rohon-Beard fibre. *c*. 16 μ, silver method, 10-day *Lampetra*; dorsal type of Rohon-Beard fibre.

destination, but there are several fine fibres in each dorsal root, which have presumably come from the dorsal ganglion-cells. The peripheral process is usually directed ventrally, but was never traced far enough to show to which functional component these early dorsal ganglion-cells belong.

Correlating System

Large Internuncial Cells. In the lateral part of the grey matter there occur some large cells whose axons run to the ventral motor tract, and whose dendrites are in clear relation with the dorsal tracts of the same and also of the opposite side of the cord (Text-figs. 6 and 7).

Dendrites to the lateral, dorso-lateral, and dorsal areas of the white matter arise from the cell-body separately. Those to the lateral area are short: those to the dorso-lateral area are longer. Dendrites to the dorsal areas have a characteristic course: they run directly dorsally, entering the white matter at an oblique angle: they then turn medially in a wide curve which keeps them clear of the Rohon-Beard cell-bodies. Most of the dendrites to the dorsal area continue medially, so that they enter the contralateral dorsal area by way of the dorsal commissure. The contralateral process runs directly across the

cord, so that the cell-body and the crossing dendrite may appear in a single section, as in Text-fig. 6. The contralateral process can also be seen in sagittal sections at 20μ, for it is quite large, and can be followed from the cell-body to the midline by adjusting the optical section. The axon enters the ventral area of the white matter, where small collaterals branch off into the ventrolateral area. The main process turns forward to run with the longitudinal fibres. Although it is now near the midline, it does not cross to

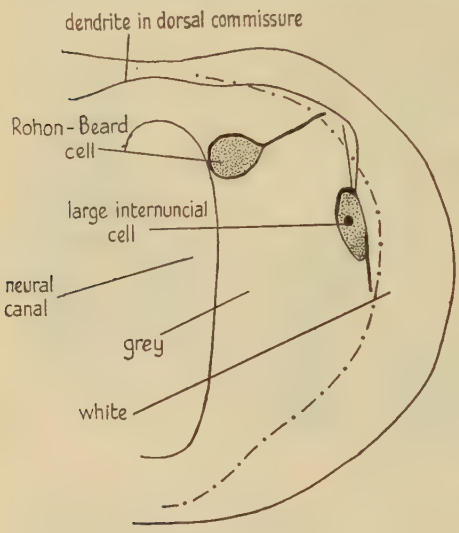


Fig. 6

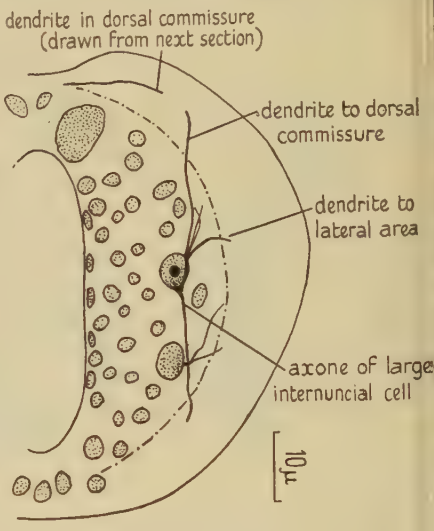


Fig. 7

Text-figs. 6 and 7. 16μ, silver method, 10-day *Lampetra*; dendrites and axons of the large internuncial cell.

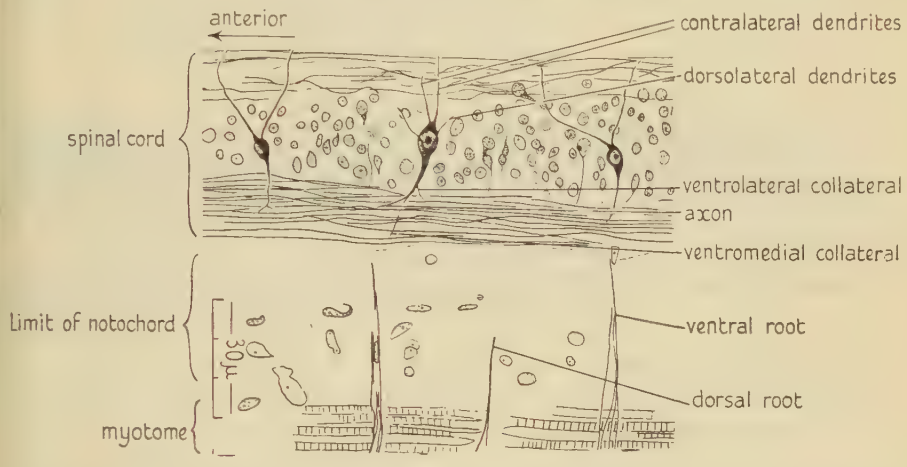
the contralateral motor areas, and no collaterals from the axon have been observed to cross (Text-fig. 8).

These large internuncial cells usually occur singly, one per segment, somewhat caudally to the origin of a ventral root, with which the axon is no doubt in synaptic relation. But the distribution of these cells is not quite so constant as might be inferred from the region figured.

Small Internuncial Cells. The Rohon-Beard and the large internuncial cells are only a small proportion of the nerve-cells in the dorsal half of the cord. Many of the remainder are neuroblasts, some of which do not appear to have yet formed any process from the cell-body: those which do have dendritic and axonic processes have not, however, achieved a definitive or constant structure, though they all appear to be developing into small internuncial cells which link the ipsilateral sensory and motor fields and which have no longitudinal process. Cell-bodies of this type of neuron lie at the dorso-lateral edge of the grey matter. There is a thick, 'protoplasmic' rather than fibrillar axon, which runs down between the grey and the white and ends on

the cell-body or immediately adjacent dendrites of a motor cell. The dendrites are short, and are usually directed into the dorso-lateral white, but they are diffuse in form and very lightly stained. It is probable that the dendrites are growing towards the incoming central fibres of dorsal ganglion-cells, which we know to be at the same stage of outgrowth. The contrast between the diffuse, almost amoeboid form of these cells, and the constant and well-defined form of the large internuncial neurons is marked.

Oblique Fibres. At fairly regular intervals along the mid-trunk region of the cord a single large fibre swings out of the dorsal funiculus and descends

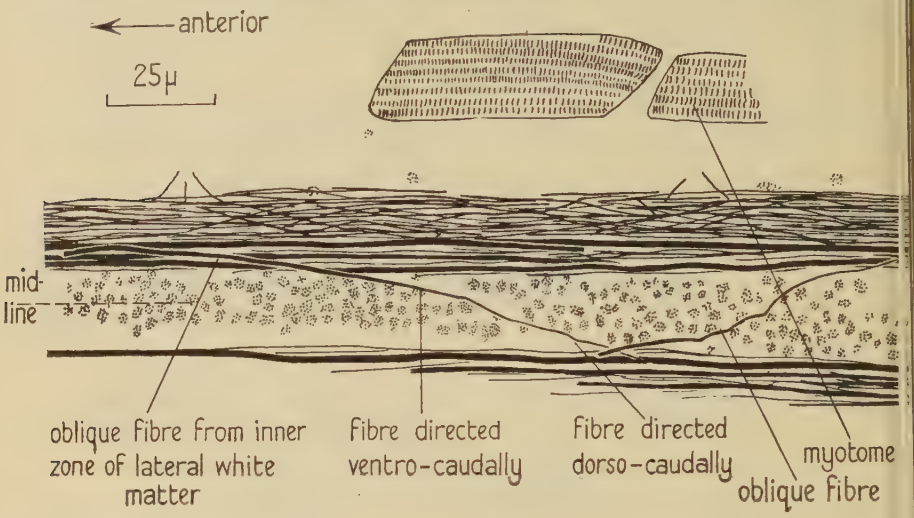


TEXT-FIG. 8. Parasagittal section, 20 μ, from a 10-day *Lampetra*; protargol silver method. Three successive large internuncial cells, showing their position relative to the motor roots.

obliquely towards the ventral funiculus: it reaches the latter about four somites more caudal than its origin from the dorsal funiculus: the fibre continues caudally in the inner and medial part of the ventral white, internal to the Müller fibres. The oblique fibres can be picked out in transverse sections, where it can be seen that during the oblique part of their course they pass medial to all the longitudinal fibres of the lateral white. In parasagittal sections the oblique fibres stand out sharply, because the other large fibres all run longitudinally in the cord. Since they are on the inner side of the white matter they necessarily pass close to at least one of the large internuncial neurons: but no synaptic relation was observed between them. Individual oblique fibres were followed towards the head in the dorsal white matter; but the cell of origin has not yet been found, nor has any large cell of different form from the Rohon-Beard cell been noticed in the dorsal white at an interval of one per four somites (Pl. Ic).

In horizontal sections of the ventral part of the cord, the oblique fibres are seen to continue in a ventral and caudal direction until they are directly medial to the Müller fibres; they may run parallel with the Müller fibres for a short distance, but they soon swing abruptly out of line, turning ventrally

and medially to reach the ventral white of the opposite side by way of the ventral commissure. Each oblique fibre turns to run parallel with the contralateral Müller fibres, but becomes rapidly attenuated and terminates (Text-fig. 9). The oblique fibres seen in horizontal section appear at fairly regular intervals, about four somites' distance separating successive fibres of the same side. Those of the two sides are not paired. Oblique fibres have not been seen in either sagittal or horizontal sections to occur in the anterior third



TEXT-FIG. 9. Horizontal section, 16μ, 16-day *Lampetra*; silver method. (Plane of section is slightly more ventral towards the lower left side of drawing.) Fibres drawn as if viewed from dorsal aspect; shows the course of two oblique fibres in the ventral commissure.

of the spinal cord: it seems probable that, at this stage of development at least, they do not occur in that region.

Cells of the Commissura Infima Halleri. In a dorso-medial position, in the most anterior part of the cord, there are on each side of the midline about twenty neurons with characteristic bipolar, spindle-shaped cell-bodies. All are oriented transversely: from the medial end of the spindle a single dendritic process crosses the midline close to the dorsal surface of the cord to end in the contralateral dorsal part of the cord. The outer end of the spindle is formed by the axon, which runs laterally into the dorso-lateral part of the white matter (Pl. IA).

These neurons can be seen in horizontal and transverse sections of 16-day prides, when they have the form described: but in 10-day prides the cells are still unipolar neuroblasts. They occur in a length of about 60μ of the cord, posterior to the choroid plexus of the medulla oblongata: the most caudal is at the same transverse level as the most anterior Rohon-Beard neurons. The diameter of the cell-body, measured across the spindle, is about one-third that of the Rohon-Beard neurons. The centre or nucleus, constituted by

these cells, lies dorsal to the ordinary cells of the dorsal grey, and median to the two dorsal funiculi, which at this level are separated from each other by this centre.

Unfortunately, the longitudinal relations of the centre are not yet known. A few of the axons can be seen to turn caudally after reaching the dorsal funiculus, while a small collateral runs anteriorly. But the details already established show that these neurons constitute by the sixteenth day a Commissura Infima Halleri and its Nucleus.

Müller Fibres. The axons of the Müller cells of the brain run toward the tail for the whole length of the spinal cord. In transverse sections of the

TEXT-FIG. 10. 12μ, 16-day *Lampetra*; silver method. The ventral commissure.

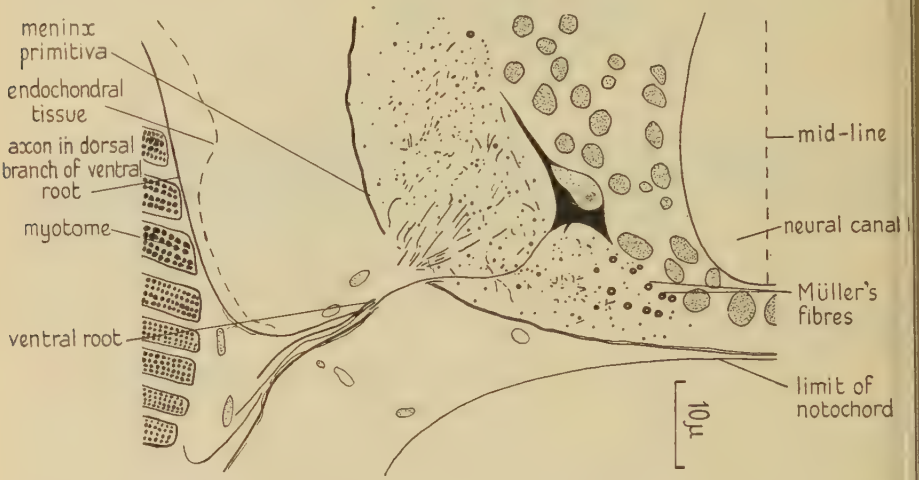
trunk they are seen below and lateral to the floor-plate cells and median to the other longitudinal fibres of the ventral funiculus (Text-fig. 10).

On each side of the cord there are about eight Müller fibres which are larger in diameter than the other longitudinal fibres of the cord, and about another eight are of the same diameter as the biggest of the fibres in other tracts. Each fibre remains on the same side of the cord as its cell of origin, and closely parallel to its fellows, so that the pattern made by the Müller fibres remains constant through many transverse sections. The fibres do not divide, nor have they been seen to give rise to any collaterals.

This description agrees with the accepted account of older animals, except that the Müllerian fibres in the young pride do not have the extraordinarily enlarged diameter found in older prides, and the tract still retains the position to be expected in a motor co-ordinating system. The cells of origin in the brain show extremely well in the present material: their form and the relations of their dendrites correspond with the figures given by Tretjakoff. The Müller cells lie in two groups, one in the midbrain and the other in the hindbrain: the dendrites of the two groups bring the cells into synaptic relation with all cranial sensory nerves except the optic and olfactory. A single large cell on each side can be seen in the present material to reach most parts of the forebrain with its dendrites, and to have an axon descending to the region of the Müller cells of the midbrain. There are optic fibres in the optic chiasma at this stage; and Studnicka and Walls (1944) have stated that there is a primitive

functional lens at this period. Presumably, therefore, the Müller fibres of the spinal cord may be affected by stimulation of any of the cranial nerves, including those of the forebrain group.

At the most anterior levels of the spinal cord a pair of the Müller fibres, apparently of similar cranial origin to the others, turns laterally and dorsally, reaches a position median to the lateral funiculus, and continues caudally, one on each side, in this position for the whole length of the trunk. This dorsalward movement of one pair of Müller fibres is very striking in the young ammocoete.



TEXT-FIG. 11. 12μ, 16-day *Lampetra*; silver method. A primary motor neuron.

Motor System

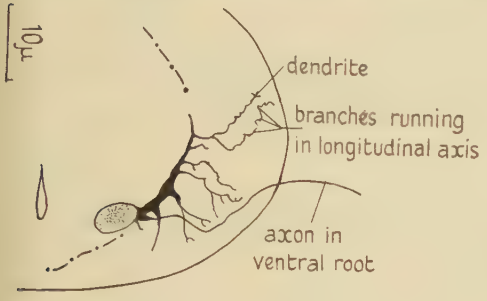
Somatic Motor Neurons. Some of the larger axons in the ventral root can be followed, in a single transverse section, peripherally to motor end-plates on the myotome and centrally to cell-bodies lying in the ventral area of the grey matter. In such cases the axon grows from the cell-body into the ventral motor tract; from the tract its course to the motor root is horizontal (Text-fig. 11). The majority of motor neurons are not so easily traced, because the cell-body and the ventral root to which it contributes are in widely separate transverse planes; but almost all the large axons can be traced from myotome to ventral root and thence horizontally as far as the ventral motor tract.

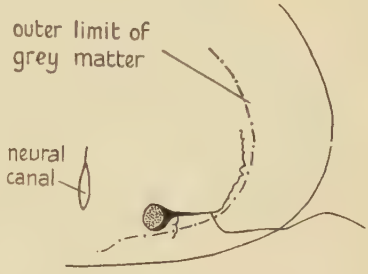
The motor neurons of the ventral grey area are of two forms. Those lying in the outer part have a 'protoplasmic' area of the cell-body on the lateral aspect of the nucleus: this area extends dorsally between the grey and the white, giving origin, on its surface against the white, to many branching dendrites which terminate on the lateral and ventrolateral surface of the spinal cord. The axon originates separately, from the outer and ventral part of the cell-body (Text-fig. 12). On entering the ventral motor tract

it turns longitudinally, giving off the peripheral fibre to the motor root as a collateral.

Neurons of the inner part of the ventral grey matter effect the same relations in the cord, but the processes from the cell-body are thin, cylindrical fibres until they reach the boundary between grey and white, when they also form a protoplasmic extension at the base of the dendrites, though it is less extensive (Text-fig. 13).

These ventral motor neurons usually have a separate process on the ventral or ventromedial surface of the cell-body (Text-figs. 10–13). This process can sometimes be traced into the ventral motor tract of the opposite side; sometimes it appears to originate as a collateral of the axon. These commissural





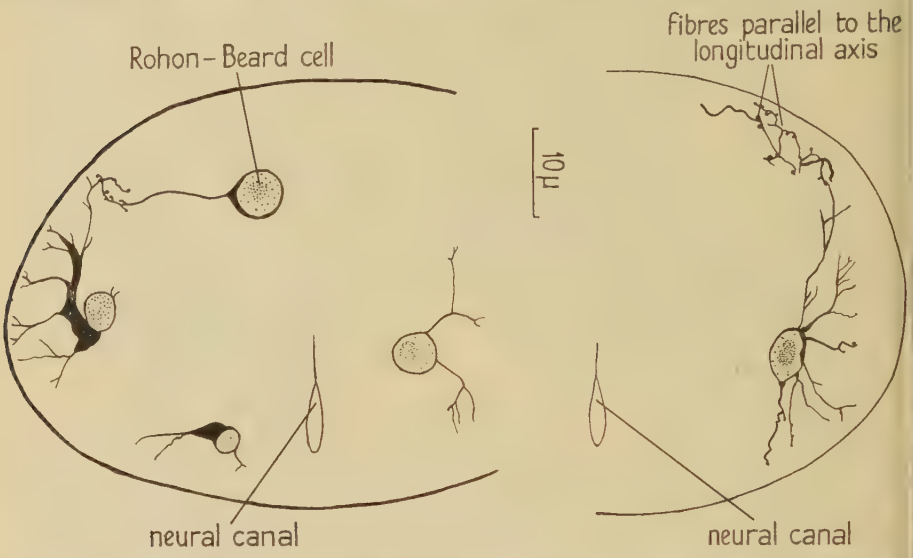
TEXT-FIGS. 12 and 13. 34μ, methylene blue method. Primary motor neurons.

fibres are best seen in cases where their whole course is through the white matter, as in Text-fig. 10. They appear to have no relation with the Müller fibres of their own side; their relation with the Müller fibres of the opposite side may be closer, but has not yet been determined. The commissural fibres usually pass ventral to both sets of Müller fibres, but sometimes they pass dorsal to those of their own side; two of this latter type can be seen in Text-fig. 16.

The neurons of the ventrolateral grey matter which are developed by this stage probably also belong to the somatic motor component, of which they would then constitute a second type. As in the case of those just described, the dendrites from neurons lying deep in the grey are thin fibres, while the dendrites of cells on the outer limit of the grey matter take origin from a broad 'protoplasmic' area of the cell-body. The dendrites reach an extensive area of the lateral funiculus, but have not yet been found to extend into the ventral funiculus. A great proportion of the branches of the dendrites run a short distance longitudinally in the lateral funiculus, so that they must represent a large part of it. Unlike the first type these neurons have some dendrites which extend dorso-laterally to come into contact with the longitudinal fibres of the Rohon-Beard cells. The axon is directed ventrally before leaving the grey matter, so that they enter the ventral funiculus close to the axons of somatic motor neurons of the first type. Instances in the second type where the cell-body, the intramedullary and the peripheral part of the axon lay in

the same transverse section have not so far been found. The motor neurons of the second type appear to be somewhat younger than the first type, and did not show up so well in the silver material, which was prepared from slightly younger embryos (Text-figs. 14 and 15).

Visceral Motor Neurons. The innervation of the splanchnic muscle of the gill-region appears to be derived entirely from the vagus nerve, which can be seen on the ventral and outer side of the anterior cardinal veins. The segmental ventral branch of each spinal motor nerve of this region passes



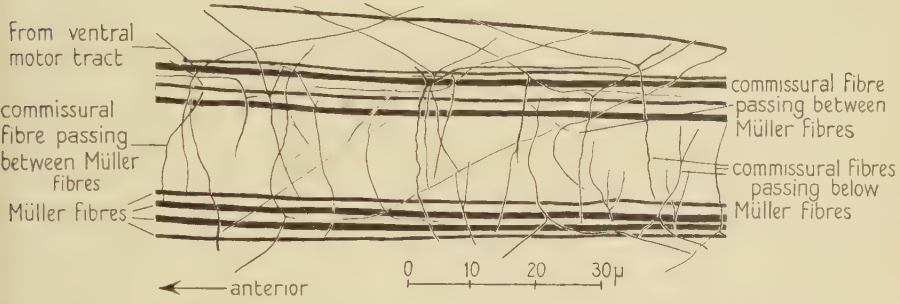
TEXT-FIGS. 14 and 15. 34μ, methylene blue method. A second type of motor neuron, in which some dendrites reach the longitudinal fibres of Rohon-Beard neurons.

close to the vagus: but the fibres of the branch remain adherent to the myotome and none appear to join the vagus nerve or to innervate splanchnic structures directly, or to reach a sympathetic ganglion: nor have sympathetic ganglia yet been identified. It is possible that a small viscero-motor component may, however, exist and have been overlooked, because the organs lying in the area between the pharynx or the intestine and the myotomes are surrounded by stellate melanophores. Consequently, in silver preparations, the black nerve-fibres are extremely difficult to follow among the melanophores. Evidently the viscero-motor component of the spinal nerves, so far as it exists at this stage, can be concerned only with the innervation of blood-vessels, and perhaps of the anal region of the gut (Dohrn, 1888). It appears improbable that the motor neurons of the ventrolateral grey belong to the viscero-motor component.

In older *Lampetra* the hypoglossal nerve is built up by the union of ventral branches from each motor root of the pharyngeal region. These branches individually enter and run a short distance with the vagus nerve before

segregating into a hypoglossal nerve (Neal, 1897; Johnston, 1905). The association between the hypoglossal and vagal nerves is certainly brought about by the backward extension of the gill-region during development. It is interesting that at the present stage of development the association has hardly commenced.

Ventral Commissure (Text-fig. 10). Some of the fibres in the ventral commissure have been described already as originating directly from motor cells of the ventral grey matter. But the great majority of the fibres crossing in the ventral commissure do not arise from a cell-body at the same transverse level, but from a longitudinal fibre of the ventral funiculus. In these cases, a longitudinal fibre swings towards the midline, passing under the Müller



Text-fig. 16. Horizontal section, 16 μ, silver method. From a region anterior to that in Text-fig. 9. The origin of the commissural fibres from the ventral tracts can be seen. (Fibres drawn as if viewed from below.)

fibres of its own side, and crosses the midline in the extreme ventral position: the fibre then becomes much thinner: it can be followed as it passes ventrally to the Müller fibres of the opposite side. Some of the fibres are too fine to be followed any farther, but others can be traced laterally and dorsally within the ventral funiculus before they also attenuate entirely (Text-fig. 16).

The crossing fibre may approach the commissure from in front or from a caudal direction. As it passes under the ipsilateral Müller fibres, it usually gives off a collateral which turns away from the midline and continues in the ventral funiculus. At the midline the crossing fibre is almost or quite in the transverse plane. It does not usually branch in the opposite funiculus. The number of crossing fibres in the ventral commissure is of the order of thirty in a length of cord equal to one somite. All, or almost all, of the fibres crossing in the ventral commissure probably originate from the somatic motor neurons, either from a process direct from the cell-body or as the termination of a longitudinal fibre in the ventral funiculus.

No outgrowth has been found from any of the floor-plate cells in the present material, at any level of the spinal cord.

In the most anterior segments of the trunk of the 16-day pride, a few of the crossing fibres in the ventral commissure appear to have a synaptic relation with the contralateral Müller fibres, to which they pass very close. These

branches on to Müller fibres are extremely minute; this detail requires confirmation on older material.

The Tail Region

The very short tail region is omitted from the present account because the anatomical relations have proved very difficult. In particular, it has not been possible to find out the relations of the Müller fibres in the tail region.

Comparison of the Spinal Cord of Adult *Lampetra* with that of the Newly Hatched Larva

Possible homologies between the neurons described here and those in the spinal cord of older animals will be discussed only briefly, because the forms of neuron to be found in the latter were the subject of furious disagreement between Kolmer, Tretjakoff, and Johnston (1910). Knowledge of the sensori-motor arc in the adult is therefore very uncertain.

It is clear from the foregoing account that the Hinterzellen of the adult must be persistent Rohon-Beard cells. Both the Rohon-Beard cell of the young pride and the Hinterzell are very large dorsally placed intramedullary cells: they occur in two rows for the whole length of the spinal cord: no other large neuron occurs near them. They both have a large fibre ascending and a large fibre descending in the dorsal funiculus. The Rohon-Beard cell has a somatic-sensory peripheral process. A similar process is found on the Hinterzell of older prides (Freud and Beccari), but is reduced or even lost in the adults (Johnston, 1902). Correspondingly, the dorsal ganglion-cells have become the predominant source of somatic sensory peripheral fibres in the adult.

The Rohon-Beard neuron has the form of a large dorsal ganglion-cell, in spite of its intramedullary position; but in the young animal it appears to combine the functions of a ganglion-cell with those of a dorsal horn cell in the somatic sensory column. As a Hinterzell in the adult it has partly lost the first of these functions.

The large internuncial neurons of the young pride are difficult to identify among the cells described in the adult or in the older larvae; but the 'Type II motor cells' of Tretjakoff's figures appear to me to be the probable homologue. The small internuncial cells probably correspond to Tretjakoff's 'amacrine' cells.

It is difficult to say which type of cell in the adult corresponds to the oblique fibres.

The somatic motor neurons have similar relations to those described in the adult by Tretjakoff (1927). But the dendrites of these neurons do not have the bizarre arborizations at this stage, which they have acquired in the adult.

The relatively immense Müller fibres of the adult correspond in the young pride to more normal-sized fibres confined to the area in which the medial longitudinal bundle is found in Gnathostomes.

Although the individual neurons of the Commissura Infima Halleri are less developed than Johnston (1910) found them in the adult, the centre and commissure as a whole has established by this stage its relation with the brain and with the spinal cord.

In short, the spinal cord of the newly hatched ammocoete does not exhibit the unusual characters of morphology and of the shape and relations of the constituent neurons which make it difficult to regard the cord of the adult as a prototype of the spinal cord of gnathostome vertebrates.

Comparison with Spinal Cord of Gnathostome Embryos

Reference is particularly made to Coghill's and to Youngstrom's accounts of *Amblystoma*. But the result would not be different if the comparison were made with earlier work on fish larvae, such as van Gehuchten's account of *Salmo* alevins (1895). Similarities between the *Amblystoma* larva and the young pride are:

1. There is a well-defined central grey and an outer white matter. The spinal cord is more or less cylindrical. The dorsal root, dorsal ganglion, and ventral root occur in corresponding positions.

2. The Rohon-Beard neurons are organized in the same relation to other neurons, occupy the same position, and have the same form. (Cf. Coghill, 1929, figs. 9, 10, and 11.)

3. The 'dorsal intercalated' neuron is probably homologous with the large internuncial cell of the young pride. The only notable difference is that the latter has not yet developed any process crossing in the ventral commissure. (Cf. Youngstrom, 1940, fig. 1, and Text-fig. 17 of this paper.)

4. Two forms of somatic motor neuron are found in both larvae. The primary motor neuron of *Amblystoma* is similar in all essentials to the motor neuron in the ventral grey of *Lampetra*: both have a 'protoplasmic' extension between the grey matter and the white and have dendrites extending through the lateral and ventrolateral white areas; in both the peripheral fibre is given off as a collateral of a fibre in the ventral motor tract; both have a process into the contralateral ventral funiculus; in both the cell-body does not usually lie at the same transverse level as the ventral root. Possibly they both have the same functional relation to a giant-fibre system descending from the brain. For the primary motor neuron of *Amblystoma* and of other anamniote Gnathostomes using the trunk-muscle as the locomotor organ, including teleosts, dipnoans, and anuran tadpoles, is in synaptic relation with Mauthner's fibre which originates in the contralateral hindbrain. In *Lampetra* the Müller fibres are derived from cells in the ipsilateral part of the brain, but the fibres appear to be developing a synaptic relation with the ventral motor neurons of the *opposite* side. If this should be established the Müller fibres might prove to have the same function in the trunk region of the lamprey as is effected by Mauthner fibres in fishes.

A more dorsal group of somatic motor neurons has been described by Youngstrom, the 'secondary' type. They also probably occur in the

other Gnathostomes of the anamniote division. They are concerned with local movements, while the primary type effect the total movements. Movements of paired limbs, independent of trunk movements, are believed to begin when the secondary type first appear at the appropriate level of the cord.

The ventrolateral group of motor neurons in *Lampetra* are probably also somatic motor, and have some characters in common with the secondary type in *Amblystoma*. They are in synaptic relation with the afferent neuron, they have apparently no relation with the longitudinal giant-fibre system, nor with the ventral tracts of the opposite side of the cord, and the axon is very slender. But the ventrolateral group have not been investigated in sufficient detail for these similarities to have much weight. Their relations with other neurons at present suggest that they are concerned with some very local form of movement.

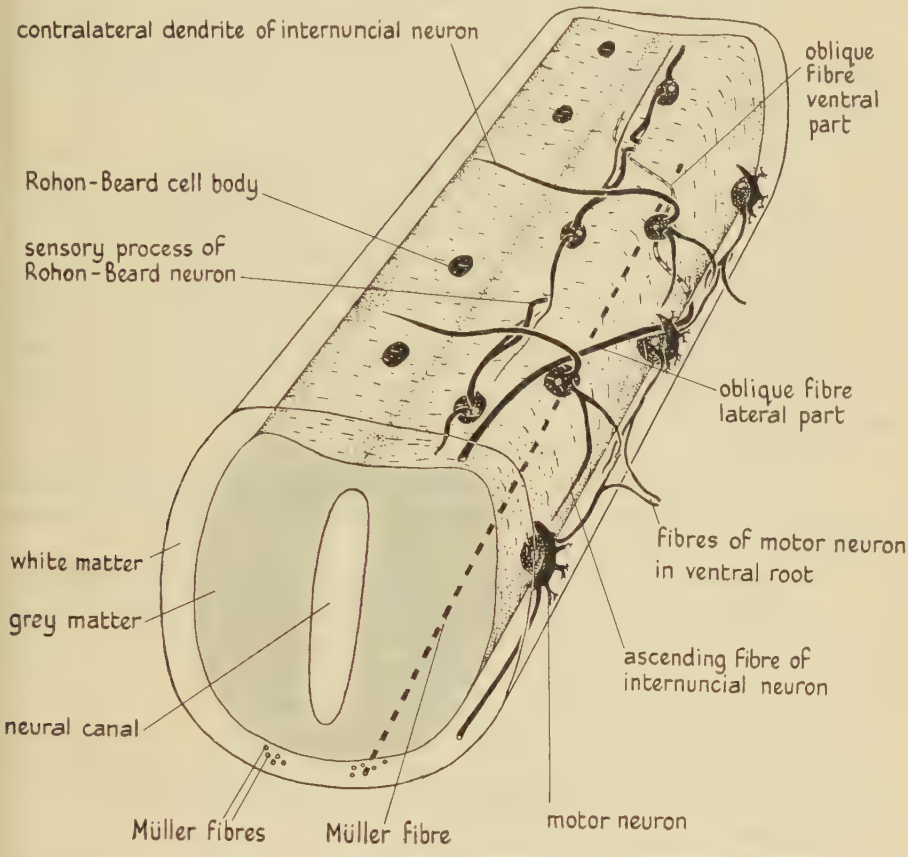
The Commissura Infima Halleri has not apparently been described in very young urodeles: it is not mentioned in Coghill's summary (1929) of his work. In amniotes the commissure is considered to correlate the viscero-sensory columns of the two sides: it was regarded as having that function in the adult *Lampetra* by Johnston (1902). But Herrick (1908) showed that in fishes the commissure has a viscero-sensory and a somatic-sensory component: in fish such as the trout the somatic-sensory part of the commissure is the more important. In the trout embryo the commissure is developed at a very early stage, before the embryo can swim, and when the viscero-sensory system of the cord has hardly begun development. It is legitimate to suppose that in the young *Lampetra* the Commissura Infima is an important part of the correlating mechanism involved in swimming movements.

The Somatic Sensori-Motor Arc in Young Pride (Text-fig. 17)

It seems clear that at this stage the Rohon-Beard cells are the chief sensory mechanism. They receive stimulation from sensory endings of very simple nature in the skin and, to a less extent, in the muscle. There are no muscle-spindles nor any proprioceptors in parallel with the myofibrils. Some Rohon-Beard neurons are entirely exteroceptive in function. Centrally, the impulses of sensory origin in the trunk pass up and down the trunk without any strict segmental organization.

The excitatory impulses can pass from the Rohon-Beard tract to the Commissure of Haller or to the Descending Trigeminal centre, thereby affecting either the other side of the trunk or the brain: or the impulse may pass to motor neurons by way of the large internuncial cells, or directly through the dendrites of the ventrolateral motor neurons, or by way of the oblique fibres. Of these paths the most developed at this stage, and that about which most detail has been elicited, is that through the large internuncial cell. But even here it is difficult to see how an apparently simple locomotor system should be related to so complex and precisely organized a system of dendrites, axons, and axon collaterals.

Consideration of the dorsal ganglion-cells, small internuncial cells, and ventrolateral motor cells is not profitable until slightly older material is available, in which these types of neuron would be more differentiated. Equally, the earliest stages in the development of the somatic sensori-motor arc are not covered in the present study.



TEXT-FIG. 17. Stereogram of spinal cord of *Lampetra*, 10–16 days after fertilization, to show the relations between some of the neurons described. The figure is constructed to show a length of cord viewed from an anterior and dorsal position.

Clearly there is already in this stage of the ammocoete a complex and precisely organized structural pattern, both of the form of the neurons and of their relation to each other. Any satisfactory description of the function of this nervous system must account for the intricacies of this pattern.

I wish to thank Professor James Gray, F.R.S., for his kind encouragement and advice on problems of neuro-embryology, and Professor J. E. Harris, Department of Zoology, Bristol University, for his helpfulness in considering the problems of function which are implicit in the description of nervous

anatomy, and also for some essential advice on problems of neurological technique. I also wish to thank Dr. J. E. Smith for the construction of Text-fig. 17. Finally, I am greatly indebted to Mr. A. R. Hockley, University College, Southampton, for the collection and preservation of most of the specimens on which this account was based.

Summary

1. A method for the study of the nervous system of vertebrate embryos by methylene blue vital staining is described. A reliable technique for rendering the preparations permanent is described.

2. An adaptation of the silver 'on-the-slide' method is given.

3. Three types of sensory intramedullary neuron are described in the spinal cord of recently hatched ammocoetes, or prides, of *Lampetra planeri*. All three are regarded as types of Rohon-Beard cell.

4. Four contemporary correlating types of cell are described in the cord: large internuncial neurons with a dendritic system which reaches the contralateral dorsal funiculus; cells of the Commissura Infima; oblique fibres, descending caudally from the sensory to the motor tracts; and small internuncial neurons with short dendrites.

5. The relations of the Müller fibres in the trunk are described in part.

6. Two types of motor neuron have been found; the more fully developed corresponds to the primary motor neuron of aquatic larvae of other anamniote vertebrates.

7. The peripheral fibres of the somatic system of the trunk are described.

8. The neurological pattern revealed is compared with that in adult *Lampetra*: the divergences from the vertebrate pattern found in the cord of the adult are not found in the young ammocoete, which in this, as in so many respects, is a good prototype of gnathostome vertebrates.

9. The probable functional pattern is compared with that found in a similar stage of *Amblystoma*.

Neurons of the correlating and motor system appear not to have been described before in ammocoetes less than 1 year old.

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DESCRIPTION OF PLATES

PLATE I

A. Transverse section showing the Commissura Infima Halleri. ×1150.
(*a*) One of the cells of the Commissure.

B. Horizontal section showing several Rohon-Beard neurons with longitudinal and peripheral processes. ×400. Compare Text-figs. 1 and 2.

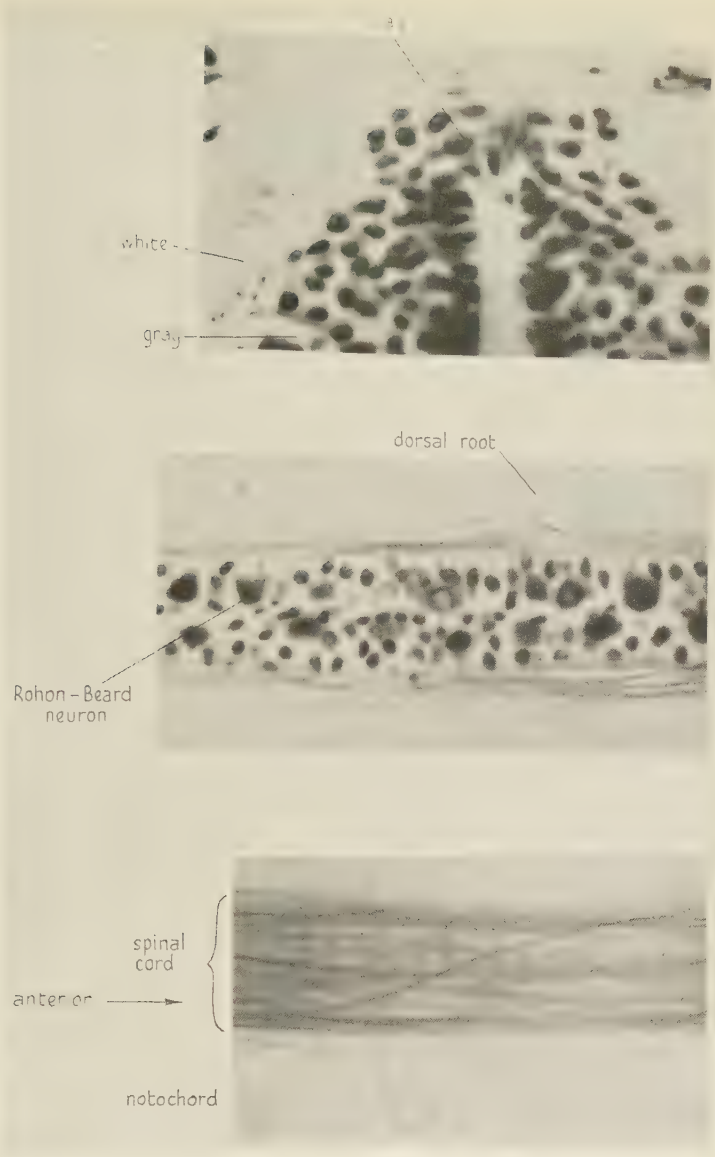
C. Parasagittal section showing oblique fibre in the lateral part of its course. ×370.

PLATE II

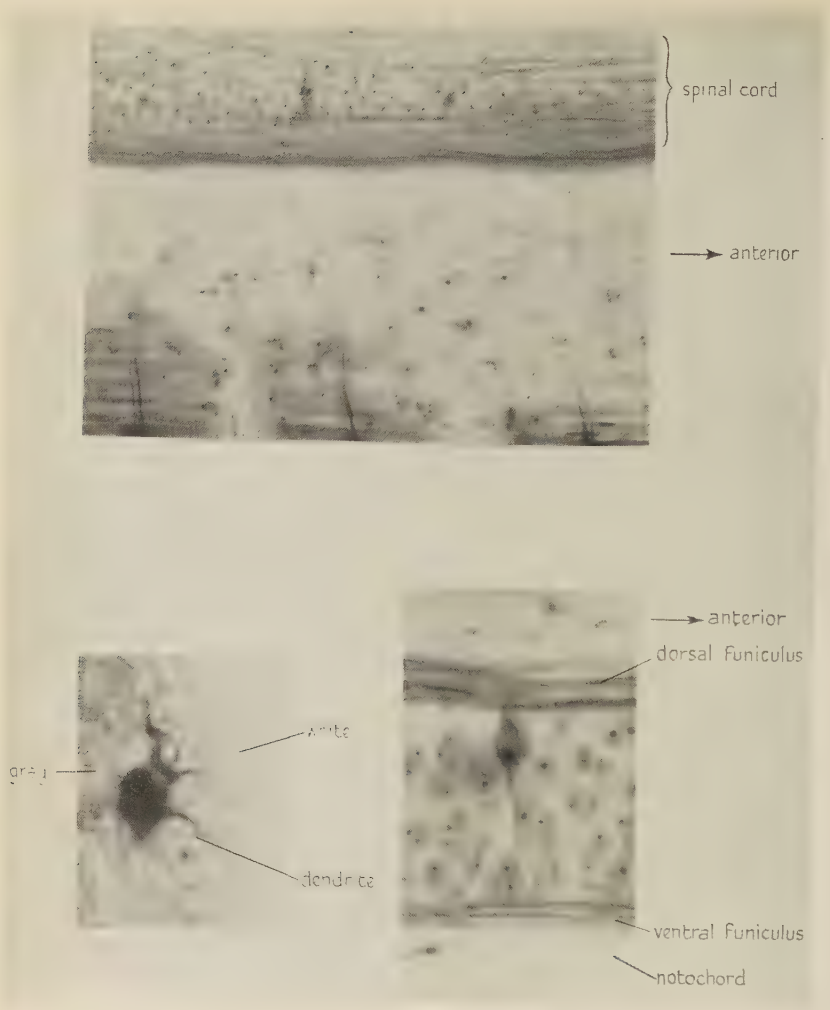
D. Parasagittal section showing relation of large internuncial cells to the segmental motor nerves and to the intersegmental sensory nerves. ×370. Compare Text-fig. 8.

E. Transverse section, 34μ, showing motor neuron stained with methylene blue. ×1700. Compare Text-fig. 12.

F. Parasagittal section showing a large internuncial cell. ×740.



WHITING—PLATE I



WHITING—PLATE II

Studies on *Lagenophrys tattersalli* (Ciliata Peritricha, Vorticellinae)

Part II. Observations on Bionomics, Conjugation, and Apparent Endomixis

BY

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CONTENTS

INTRODUCTION AND PREVIOUS WORK

THE present paper records the continuation of work on *Lagenophrys tattersalli* Willis, a marine, vorticellinid ciliate found epizoic upon the gill-plates of the amphipod crustacean *Gammarus marinus* Leach (Willis, 1942).

The existence of conjugation ('Copulation', Awerinzew, 1936) in *Lagenophrys* has been known almost from the creation of the genus. Nevertheless, previous accounts of the process are very fragmentary, a result of the acknowledged technical difficulties in studying conjugation in an epizoic, loricate protozoan. In general form, the conjugation of *Lagenophrys* resembles that of other Vorticellinae, and is of the dimorphic type, involving the association of micro- and macroconjugants (cf. Text-fig. 3A). The microconjugant was first observed by Stein (1851). Ubisch (1913) has given the fullest account of the formation of the microconjugant (named by her, variously, as a

'Conjugationsschwarmer' or 'Mikrogamet'). According to this description, based mainly on the freshwater species *L. platei*, the microconjugants are formed by the divisions of an organism produced by *budding* from a normal vegetative individual. Unfortunately the term 'Conjugationsschwarmer' is also applied by Ubisch to this bud. Awerinzew (1936) distinguished two types of microconjugant ('grossen' and 'kleinen' Gameten) in an unnamed, freshwater lagenophryid which was found in West Africa on the crustacean *Telphusa*. These microconjugants are described as being formed from two types of organisms (respectively, the 'grossen' and 'kleinen' Gametocyten) produced by the unequal *fission* of a normal vegetative individual. Since the terminology of Ubisch and Awerinzew is both confusing and inexact, the term 'protoconjugant' is applied in the present work to the organism which divides to form the microconjugants (cf. Text-fig. 2A–C). In the study of *L. tattersalli* special attention has been paid to the formation and subsequent division of the protoconjugant, and a comparison has been made between protoconjugant formation and first-type division (as previously described, Willis, 1942).

In previous work on *Lagenophrys*, little attention has been paid to the nuclear phenomena of conjugation. Awerinzew (1936) alone gives a brief description of the formation of the pronuclei and the synkaryon. Similarly, there is no record of the divisions by which the macronuclear Anlagen, formed from the synkaryon, are distributed among the lineal descendants of the synconjugant (exconjugant). For this reason special attention has been paid to these distributive divisions in *L. tattersalli* where they have an added interest at the period of ecdysis, when they conform to the pattern of a special type of obligatory division (second-type division, Willis, 1942).

There is no record of any endomictic process in *Lagenophrys*, although it may be questioned whether some of the stages figured and described by some earlier workers are not, in fact, stages in endomixis. The difficulty in distinguishing between possible endomixis and conjugation consists in (i) the relatively early fusion of the two partners in conjugation, thus obscuring the distinctive form of the process, and (ii) the fact that the organisms cannot be isolated and cultured away from the host. No attempt has been made in previous work to follow the alternative method of study, namely to trace the behaviour of known individuals upon isolated gill-plates subjected to continual irrigation. The latter method has been employed in the present work.

In conclusion attention must be drawn to certain descriptive terms used in the following account. The surface of the organism applied to the gill-plate, and the free surface, are referred to as the lower and upper surfaces respectively. The diameter passing through the middle of the lorica mouth (oral region) is distinguished as the main diameter, with oral and aboral extremities. The main diameter, together with the diameter at right angles to it, divides up the body into quadrants which are referred to as the right and left oral, and the right and left aboral quadrants.

Methods and Material

The occurrence and collection of the host and the method for examining living material for prolonged periods by means of Kitching's (1934) irrigation apparatus have been described previously (Willis, 1942).

The difficulties in studying conjugation in *Lagenophrys* may be overcome, in part, by removing the gill-plates from the host, and setting up preparations for continuous irrigation under the microscope. The outline of the gill-plate and the position of the epizoites may then be recorded by means of a camera lucida. In this way the behaviour of conjugants (and other stages) can be followed for periods ranging from 3 to 6 days, and the early phases of conjugation can be distinguished from endomictic or other processes of nuclear reorganization. The sequence of observations on living organisms is interrupted by the first distributive division of the synconjugant, since, as yet, no means has been devised for following the swarmer after its liberation from the parent lorica.

The sea-water used for culturing the hosts, and for the irrigated cultures, was renewed every third day by samples brought directly from the shore at Swanbridge, on the Bristol Channel, where the material was collected.

Material was extracted from the cultures at various stages and fixed in Schaudinn, Champy, or mercuric chloride plus acetic acid for later cytological study.

Since the presence of a lorica made it difficult to obtain an even differentiation, regressive stains, like Heidenhain's iron-alum haematoxylin, were not wholly satisfactory. Diluted Delafield's haematoxylin (1 part stain to 5–10 parts of distilled water), used progressively, gave good pictures of the nucleus, especially in metamorphosing forms. In most cases, however, Feulgen's 'Nuclealfärbung' proved to be the most satisfactory process for demonstrating the nuclear material sharply and evenly. Despite the value of the Feulgen process for nuclear studies, an unqualified reliance upon the method in the study of the complex nuclear phenomena of conjugation does not appear justifiable at the present time. As a result, to assess the value of the method, the Feulgen preparations have been compared, at all critical stages, with preparations made with orthodox nuclear stains. From this comparison, it is clear that the chromatin (basichromatin) is stained energetically by the Feulgen method until the final dissolution of the necrochromidium.

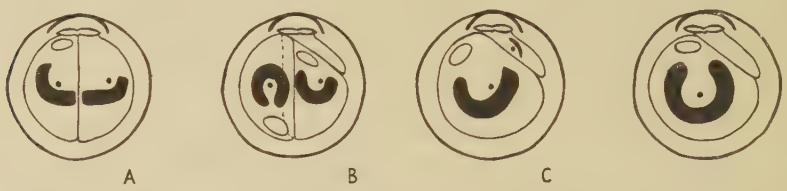
To apply the Feulgen method, the gill-plates were taken, after fixation in mercuric chloride plus acetic acid, and transferred to a little sieve made of cheese-cloth tied around a glass ring (diameter 1 in., depth $\frac{1}{2}$ in.). In this way the preparations can be removed from one fluid to another with the minimum loss of time. This is a great advantage in controlling the critical hydrolysis in N.HCl. Despite the presence of a lorica, the optimum time for the latter is 4 minutes, as in the usual technique for sections of material fixed in mercuric chloride plus acetic acid (Gatenby and Painter, 1937). Some of the preparations were counterstained in light green to give a sharp differentiation of the background.

Estimates of the percentage of the infection transferred from one instar to another of the host were made by taking the moulted cuticles from isolated individuals, and counting the number of empty loricae; the new instar was then killed, after an interval of 3 days, and the new infection counted.

The figures were made with an Abbe camera lucida with a Zeiss oil immersion lens ($\frac{1}{12}$ inch, N.A. 1·30).

Bionomics

Like all species of its genus, *L. tattersalli* is a sessile epizoite. It is restricted to the gill-plates of its host, and occurs most abundantly upon the inner



Text-fig. 1. *Lagenophrys tattersalli*. Diagrams illustrating various modes of the divisions occurring between moults and at ecdysis (based on Willis, 1942).

A. 'First-type' division. This normally occurs in the inter-moult period and produces a swarmer (on the left) and a residual organism. The swarmer escapes while the residual organism remains behind in the lorica (thick black outline), grows, and repeats the 'first-type' division process until the ecdysis of the host. B. Mode *a* of 'second-type' division, involving the rapid succession of 'first-' and 'second-type' divisions. The latter is unequal and produces a small residual organism. Two swarmers are thus produced and both escape. The small residual organism remains in the lorica and degenerates. Occurs at ecdysis. C. Mode *b* of 'second-type' division involving the unequal fission of entire organism (i.e. without an immediately preceding first-type division). The micronucleus divides equally, the macronucleus unequally. Occurs at ecdysis. D. Mode *c* of 'second-type' division. The nuclei do not divide. Occurs at ecdysis.

Macronucleus as a broad band (black), micronucleus as a dot (black), peristome as an ovoid outline in swarmer, lorica as a heavy outline. Ciliation omitted.

surfaces of these structures. The reproductive bodies, or swarmers, are 'hypotrichous' forms, adapted for moving over surfaces. They do not appear to become pelagic at any time. From the surface-dwelling habits of the swarmers and the fact that the entire colony is removed periodically by the ecdysis of the host arise two unusual problems for *L. tattersalli*: (i) the problem of maintaining the infection from instar to instar of the host, and (ii) the problem of securing the initial infection of the host. No attention has been paid to these problems in previous work on the genus.

(i) The onset of ecdysis in the host is correlated with the occurrence of second-type divisions of the protozoon (Willis, 1942). Each of these produces a normal swarmer and a small residual body. The various modes of second-type divisions and their relationships to the first-type divisions normally occurring in the inter-moult period are set out diagrammatically in Text-fig. 1. Since all the organisms composing the infection respond to the onset of ecdysis in this way, the whole colony is mobilized when the old cuticle is shed.

The swarmers may then be observed moving freely over the surface of the host. Access to the surface of the underlying new cuticle is provided by splits which appear in the old cuticle before the latter is finally shed. The transference of the infection is not complete, but appears to be about 50–70 per cent. effective. The influence of the obligatory, second-type divisions upon the form of the distributive divisions of the synconjugant is discussed later (*see* Conjugation).

(ii) The initial infection of the host by *L. tattersalli* takes place during the breeding periods of the former. During these periods some of the swarmers emerging from loricae on the gill-plates of female hosts appear to migrate into the brood-pouch which is developed by the latter during breeding periods. If hatching of the eggs contained in the pouch has already occurred, some of the migrating swarmers settle down on the gill-plates of the young gammarids. The brood-pouch is formed by the overlapping oostegites occurring on the anterior thoracic appendages of the female host, and since these oostegites lie close to the heavily infected inner surfaces of the anterior gill-plates of the parent amphipod, a direct pathway is provided for the migration of the swarmers into the brood-pouch. The actual penetration of the swarmers into the pouch has not been observed, but inspection of the gill-plates of young gammarids extracted from the pouch shows that a high proportion are always infected. This initial infection is always low and rarely consists of more than 4–6 specimens of *L. tattersalli* upon a single young gammarid. It follows from this observation, that the colonies of *L. tattersalli* on the gill-plates of a single host are likely to be highly clonic, i.e. to consist of the descendants of the relatively few swarmers which transmit the infection from host to host.

Conjugation

Formation and Division of the Protoconjugant

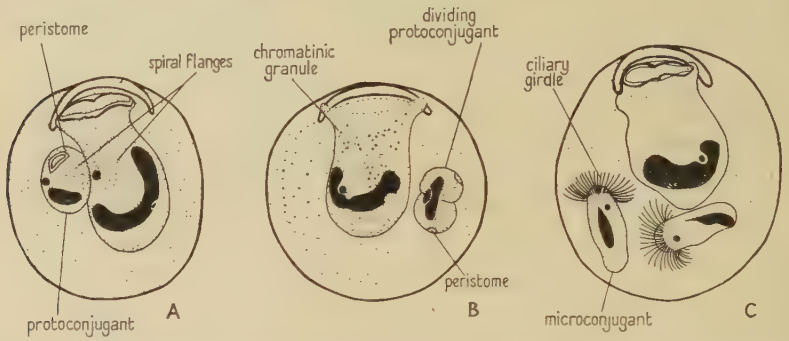
Conjugation in *L. tattersalli* resembles that of other Vorticellinae and involves the association of two dissimilar conjugants—a larger, non-motile macroconjugant and a smaller, motile microconjugant (Text-fig. 3A). Before the attachment of the microconjugant the macroconjugant resembles a normal vegetative organism in its general morphology, and in the apparent characters of the nucleus. On the other hand, the microconjugant is a specialized organism formed by the binary fission of a protoconjugant (Text-fig. 2A–C).

The protoconjugant is formed by the unequal fission of a vegetative organism (Text-fig. 2A). The plane of cleavage passes through the left oral and aboral quadrants, parallel to the main diameter. In first-type division, cleavage is along the plane of the main diameter.

The first stage in division is the movement of the micronucleus along the anterior border of the macronucleus to a point about two-thirds to three-quarters of the length of the macronucleus from its right extremity. This point marks the position of the future cleavage plane. In first-type division

the micronucleus moves in a similar direction to a point midway along the anterior border of the macronucleus.

The adoral spiral of the parent becomes contracted and the cilia and undulating membranes disappear. Before cleavage is completed structures which resemble the peristome, vestibular cavity, and spiral flange of the early swarmer (Willis, 1942) appear in the protoconjugant (Text-fig. 2A). In the swarmer these structures are formed by the division of the parental peristome, vestibular cavity, and adoral spiral, respectively. There is no con-



TEXT-FIG. 2. Stages in microconjugant formation. (×480.) A. Late stage in protoconjugant formation. B. Division of the protoconjugant. From the lower surface. C. Two mature microconjugants within the lorica. Ciliation not visible in the parent organism, and may be absent at this stage. (Feulgen preparations.)

clusive evidence to show whether the peristome, vestibular cavity, and spiral flange of the protoconjugant arise *de novo*, or by division, from the parent.

There is no evidence, in *L. tattersalli*, for the formation of the protoconjugant by budding, as described by Ubisch (1913) for *L. platei*. In the unnamed species described by Awerinzew (1936) the large protoconjugants are formed by a cleavage resembling that which invariably occurs in *L. tattersalli*. On the other hand, the small protoconjugants are said to be formed by a cleavage which runs obliquely across the left aboral quadrant. There is no indication of this type of cleavage in the formation of the protoconjugant in *L. tattersalli*, nor of any dimorphism in size among the protoconjugants. Neither Ubisch (1913) nor Awerinzew (1936) appears to have observed any cytoplasmic differentiation of the protoconjugant which may be compared with that described above for *L. tattersalli*.

At the end of cleavage the protoconjugant separates from the parent organism. At a comparable stage in the development of the swarmer free cilia are formed upon the spiral flange; this never occurs in the protoconjugant. The latter also differs from the mature swarmer in the absence of a sucker cavity and ciliary girdle on the lower surface.

After a short period of free existence the protoconjugant divides within the lorica (Text-fig. 2B) to produce two organisms which transform directly into the functional microconjugants. Before this division the micronucleus takes

up a position midway along the peristomal border of the macronucleus. The cleavage plane passes through this point and the middle of the peristome, and thus, in its general orientation, resembles the cleavage plane of first-type division. In each young microconjugant, a peristome and a small spiral flange are present. Similar structures were not observed by Ubisch (1913) nor by Awerinzew (1936) in the microconjugants of the species studied by them. It is likely that the peristome and the spiral flange are formed by the division of the comparable structures in the protoconjugant, although the evidence for this is not complete.

After the separation of the two young microconjugants, the peristome and the spiral flange disappear in each, and both organisms become elongated (Text-fig. 2c). A ciliary girdle develops around the apical extremity of each microconjugant, in contrast to the swarmer, where the ciliary girdle develops on the lower surface.

It may be concluded from the above account that in *L. tattersalli* the microconjugants are formed by two successive divisions, each of which is comparable, at least in the orientation of the cleavage plane, to first-type division.

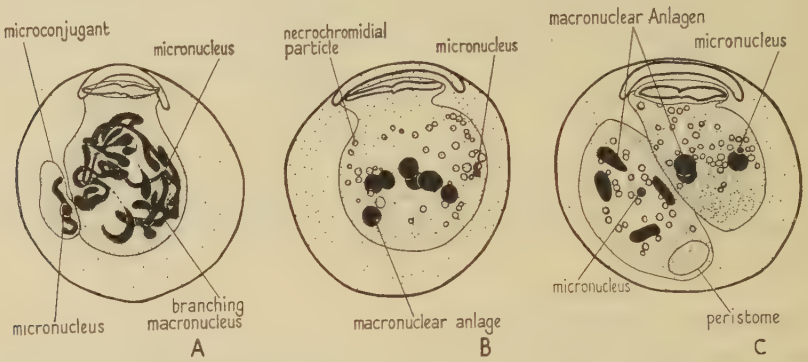
Behaviour of the Microconjugant

After the formation of the ciliary girdle the microconjugants remain within the lorica of the parent for varying periods (in one case for 2 days) before finally escaping. They usually show slow rotating movements. In those cases in which the formation of the microconjugants was traced in life from the protoconjugant, the sequence of observations ended with the escape of the microconjugants through the mouth of the lorica. In many cases, however, the organisms died before escaping. As a result the possibility that some of the microconjugants may fail to escape, and may conjugate with the parental organism, cannot be excluded. In view of the observed escape of some of the microconjugants this conclusion may appear rather fine-drawn. Nevertheless, it is enhanced by the following facts and considerations: (i) The microconjugants are pelagic organisms and are not adapted for moving over surfaces. It is thus difficult to see how they can maintain themselves on the gill-plates while exposed to the respiratory current of the host. It can hardly be supposed that any chemical attraction is exerted upon them by the macroconjugant, since any substance diffusing from the latter would not remain localized. (ii) In a high proportion (about 50 per cent.) of all preparations of early conjugating stages the functional microconjugant is accompanied, in the lorica, by a degenerating body which is clearly comparable, in many cases, to a microconjugant. In view of the above consideration (i), it seems probable that, in such cases as these, the two microconjugants formed from a protoconjugant have been retained within the lorica, and that one has become a functional microconjugant while the other degenerates. (iii) In *L. platei* Ubisch (1913) states that the microconjugant enters the macroconjugant by boring through the lorica. No evidence for this mode of entry has been found

in any living or fixed specimen of *L. tattersalli*. From (i), (ii), and (iii) above it seems reasonable to conclude that the possibility of automictic (paedogamic) conjugation is supported by circumstantial evidence. Among the peritrichous ciliates, paedogamic conjugation has been observed by Enriquez (1907) in *Opercularia*.

Nuclear Phenomena in the Micro- and Macroconjugants

In living material the first sign of impending conjugation is the rapid darting movement of the microconjugant towards the surface of the left



Text-fig. 3. Stages in conjugation up to the first distributive division. Ciliation omitted. (×480.)

A. Early stage in the association of the conjugants. The macronucleus of the macroconjugant has the branching form which is developed shortly before its fragmentation. B. Mature synconjugant. The micro- and macroconjugants have fused. C. Appearance of the first distributive division in the inter-moult period. (Feulgen preparations.)

aboral quadrant of the macroconjugant. Although there is no visible differentiation of the macroconjugant in this area, the fact that the movements of the microconjugant are always directed towards it leads to the supposition that it must exert some attraction. It is physically possible for the microconjugant to attack any point on the surface of the macroconjugant. The movements of the microconjugant are brought about by the concerted, backward sweep of its cilia.

The microconjugant eventually becomes attached to the macroconjugant by its apex, and not by its side. This is in contrast to the behaviour of the smaller organism produced in apparent endomixis (*see* Observations on Apparent Endomixis). The cilia of the microconjugant later disappear.

The cytological study of specimens at this early stage shows that there are no preparatory nuclear changes in either conjugant. This observation is of some importance, since Ubisch (1913) concluded that in *L. platei* there were preparatory nuclear changes in the macroconjugant, before the union of the latter with the microconjugant. The evidence for this conclusion is criticized later (*see* Metamorphosis and the Later Distributive Divisions).

After the disappearance of the cilia from the microconjugant the protoplasm of both conjugants becomes confluent at the point of attachment. The

micronucleus of each conjugant moves towards the point of confluence and undergoes a series of divisions. The divisions are synchronous in both conjugants, and are exactly comparable to those observed by Awerinzew (1936) in *Lagenophrys* sp. and to the maturation divisions of other Vorticellinae. In *L. tattersalli*, during the maturation divisions, the chromatin appears finely dispersed through the substance of the nuclei and no chromosome-like structures are visible. After the exchange of the migratory pronuclei, a synkaryon is formed in the macroconjugant. No synkaryon was observed in the microconjugant. The nuclear behaviour of the two conjugants ceases to be synchronous after the exchange of the pronuclei. In the microconjugant the latter quickly degenerate and disappear. This degeneration is accompanied by the absorption of the microconjugant by the macroconjugant. In view of this it seems preferable to describe the macroconjugant, subsequently, as a syn- rather than ex-conjugant. Further, in view of the fusion of the conjugants and the formation of only one functional synkaryon there is some justification for describing the sexual process of *Lagenophrys* as copulation (as defined by Doflein, 1929), rather than as conjugation.

After the attachment of the conjugants the macronucleus of the macroconjugant undergoes a remarkable transformation which has not been observed in any other species of *Lagenophrys*, and which has no exact counterpart in other ciliates (Text-fig. 3A). The macronucleus throws out branching processes which appear to become drawn around in the cytoplasm. In this process parts of the branches become attenuated and finally break, so that large separated fragments of the macronucleus become dispersed throughout the cytoplasm. At this stage there occurs a further fragmentation of the macronuclear substance and, as in conjugation and endomixis in other ciliates, an extensive necrochromidium is formed. This consists of a large number of small vesicles, each with a cortex of chromatin which surrounds a clear space.

The Maturation and the First Distributive Division of the Synconjugant

After syngamy the synkaryon moves into a central position and undergoes three successive divisions to form eight nuclei (as in *Vorticella*, Maupas, 1888, and *Carchesium*, Popoff, 1908). Seven of these become macronuclear Anlagen, one forms a micronuclear Anlage. Before dividing the synkaryon increases in size and becomes a somewhat ovoid vesicle. In Feulgen preparations, this is seen to consist of a pale-staining matrix of finely dispersed chromatin containing larger granules which stain intensely.

In most cases the divisions of the synkaryon occur in rapid succession and are synchronous. Therefore, in later stages of maturation, when the chromatin content of the Anlagen increases, the latter differentiate uniformly. The micronuclear Anlage later decreases in size to the dimensions of a normal micronucleus and its chromatin becomes condensed, the entire Anlage appearing progressively more homogeneous and deeply staining after Feulgen. In the mature macronuclear Anlagen the chromatin appears coarsely granular

and is arranged in a network. The general appearance of the mature synconjugant is shown by Text-fig. 3B. The macronuclear Anlagen and the micronucleus usually take up a position which roughly corresponds to the positions of a macro- and micronucleus in a normal, vegetative organism.

In certain cases the successive divisions of the synkaryon appear to occur less rapidly and without synchronization. This condition is indicated by the small number of maturing synconjugants in which the members of each set of macronuclear Anlagen show variation in size, and in chromatin concentration.

Between moults the first distributive division of the mature synconjugant is always a first-type division which is normal in all but the condition of the macronuclear material. The micronucleus moves into a central position on the main diameter and determines the position of the future cleavage. The seven macronuclear Anlagen become segregated into groups of three and four. Usually the group of four Anlagen passes to the swarmer (Text-fig. 3C), the remainder being retained in the residual organism. The lipoid and fatty reserve materials are either divided equally or segregated in the swarmer, as in normal, non-distributive first-type divisions (Willis, 1942). The food vacuoles are retained in the residual organism while the necrochromidium is divided equally between the swarmer and the residual organism. Since all these inclusions (i.e. fat, lipoid, food vacuoles, and necrochromidium) are uniformly dispersed throughout the cytoplasm before division, it is clear that simple cleavage cannot account for the independent segregations which have been observed. As yet no explanation can be given of this feature of the division process. At the end of cleavage the macronuclear Anlagen of the swarmer become elongated. This change of form seems to be comparable to the elongation and attenuation of the macronucleus in the non-distributive swarmer of asexual reproduction (Willis, ibid.).

At ecdysis the obligatory second-type division cuts across the normal progress of maturation, and may considerably modify the character of the first distributive division. In consequence conditions are often observed which appear to have no parallel among the free-living ciliates. A comparison with the behaviour of other ciliates which are epizoic upon arthropods would be of interest, but this is impracticable owing to the lack of previous work on the responses of the epizoites to the ecdyses of their hosts.

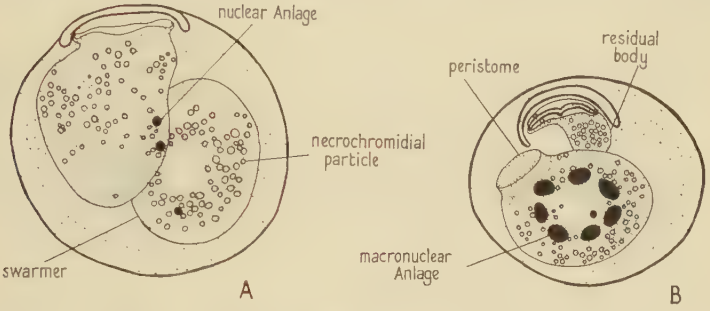
The behaviour of the synconjugant at ecdysis may be considered in two categories, (i) when the synconjugant is mature at ecdysis, and (ii) when it is immature at ecdysis.

(i) The mature synconjugant has been found to respond to ecdysis by modes *a* and *c* of second-type division.

In mode *a* responses (i.e. when there is a rapid succession of first- and second-type divisions) the first-type division is usually comparable to that which normally occurs in the inter-moult period. The following second-type division then produces a swarmer with three Anlagen, and a small residual organism.

In mode *c* responses (Text-fig. 4B) the seven macronuclear Anlagen become grouped in a circle which occupies a position similar to that occupied by the horseshoe-shaped macronucleus of an asexual swarmer. The micronucleus lies centrally, within the macronuclear Anlagen. The latter are ovoid in the distributive swarmer. The small residual organism contains a small portion of the necrochromidium which is cut off from the main mass by the cleavage plane.

(ii) In cases where the synconjugant is immature at ecdysis the second-type divisions may again be by modes *a* and *c*. The first-type division of an



TEXT-FIG. 4. Stages in second-type division at ecdysis, showing modifications of the first distributive division. Ciliation omitted. (×480.)

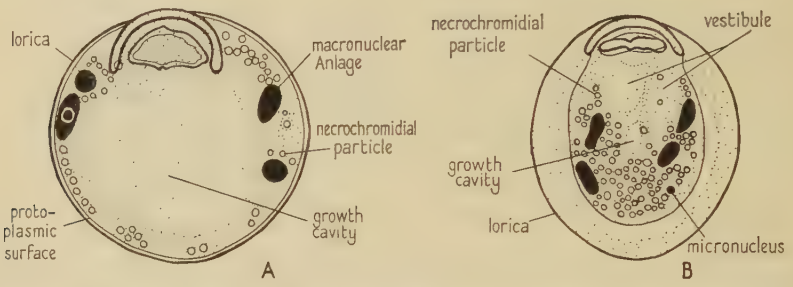
A. First-type division of a mode *a* response in which one of the divisions of the synkaryon appears to have been cytoclastic. From the lower surface. B. Mode *c* response. (Feulgen preparations.)

incompletely developed mode *a* response is shown by Text-fig. 4A. The swarmer possesses one condensed nuclear Anlage, while two similar bodies are present in the residual organism. These nuclear structures are smaller than the macronuclear Anlagen of a maturing synconjugant. They resemble normal micronuclei in size, but contain less chromatin. In this specimen it seems clear that one of the divisions of the synkaryon has become *cytoclastic*, and has determined the equal, binary fission of the synconjugant. Unfortunately it has not been possible to trace the later behaviour of the swarmers produced by this unusual type of distributive division. It seems reasonable to suppose that the nuclei which are present in such swarmers (and those of the residual organism as well) later divide further, as if they were part of the normal series of nuclei produced by the synkaryon during the intermoult period.

Metamorphosis and the Later Distributive Divisions

Owing to the impossibility of following the path of the swarmer, the lineal descendants of the marked forms kept under observation up to the period of the first distributive division could not be traced. The following account is therefore based upon the seriation of various fixed stages.

The metamorphosis of forms with four Anlagen has been observed frequently (Text-fig. 5A, B). The figured specimens show the metamorphosis of swarmers produced by the first distributive division. The reorganization of the nuclear material produced by conjugation is without effect on the complex process of metamorphosis (as previously described, Willis, 1942), except that (i) the micronucleus may take up various positions, in contrast to normal metamorphosis, when it lies embedded in the right limb of the macronucleus, and (ii) the greatly distended growth cavity appears to exert less pressure upon the scattered macronuclear Anlagen than upon the normal macronucleus, which becomes greatly attenuated during metamorphosis (Willis, 1942).



TEXT-FIG. 5. Early (A) and late (B) stages in the metamorphosis of swarmers formed by the first distributive division. Ciliation omitted from B. (×480.) (Delafield's Haematoxylin, after Schaudinn fixation.)

In vorticellinids generally, three generations are required to distribute the macronuclear Anlagen of the synconjugant. As a result eight organisms are produced, each with a single macro- and micronucleus. In a number of cases, however, forms are found in which the morphologically single macronucleus shows a gross, moniliform character, with two, three, four, or even seven lobes, i.e. with the number of lobes corresponding to the numbers of discrete Anlagen found in normal distributive stages. This may indicate that in certain cases the Anlagen become fused together. If this is so, then certain apparently normal asexual divisions may in fact be distributive.

The necrochromidium persists throughout the series of distributive divisions and, in some cases, even later. The constituent particles retain their vesicular character.

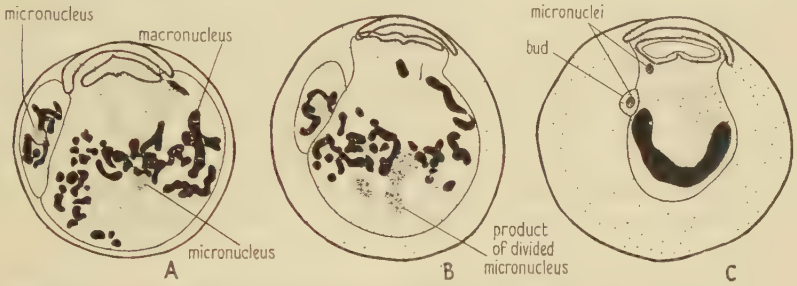
In *L. platei* Ubisch (1913, Text-fig. 44) describes and figures certain specimens which bear a close resemblance to some of the distributive generations of *L. tattersalli*. Ubisch, however, did not consider the distributive divisions, and interpreted these specimens as stages in the maturation of the macroconjugant in readiness for conjugation (Conjugationserwartung). In view of the absence, in *L. tattersalli*, of nuclear preparation in the macroconjugant before its union with the microconjugant, it seems highly probable that the 'Conjugationserwartung' of Ubisch is based upon specimens produced by the distributive divisions of a synconjugant.

Observations on Apparent Endomixis

The Normal Process

The analysis of colonies showing 'epidemic' conjugation always reveals a small number of forms which, to a superficial view, appear to be undergoing conjugation (Text-fig. 6A, B), but which, on closer inspection, show certain differences from the normal mode of that process.

These forms appear to consist of two unequal organisms which never separate. The smaller organism shows some resemblance to a protoconjugant which has failed to separate from its parent. It differs from a protoconjugant in the following characters: (i) the cleavage plane, by which it is formed, runs



Text-fig. 6. A. Early stage in apparent endomixis, showing the formation of the larger and smaller organisms (the smaller organism is on the left-hand side). B. Later stage in apparent endomixis. The micronucleus of the smaller organism is undivided; the micronucleus of the larger organism has undergone two successive divisions. C. Late stage in a process of anomalous micronuclear division. Ciliation omitted. All figures ×480. (Feulgen preparations.)

obliquely across the left oral quadrant of the parent, and (ii) the peristome, vestibular cavity and dexiotropic spiral flange of the typical protoconjugant are absent. The smaller organism differs from a microconjugant in that (i) it is not formed by the binary fission of a protoconjugant, and (ii) it is attached along the whole of its side to the left oral quadrant of the larger organism, and not by its apex to the left aboral quadrant, as in the case of the attachment of the micro- to the macroconjugant in conjugation.

Morphologically, the smaller organism may be regarded as a protoconjugant which has failed to separate from the parent, and which has undergone no further differentiation.

Differences from the process of conjugation are also shown by the behaviour of the nucleus in the larger and smaller organisms. In the first place, the micronucleus of the smaller organism, although it becomes enlarged and vesicular as in the first stages of the maturation of the micronucleus of the microconjugant, does not develop further, and appears to degenerate without division. In the larger organism, the micronucleus takes up a central position. In a macroconjugant, on the other hand, the micronucleus becomes eccentric and moves towards the area of confluence with the microconjugant. Later, the micronucleus of the larger organism becomes enlarged and vesicular, and

divides twice to form four daughter nuclei (Text-fig. 6B). There is no evidence that a third division occurs, as in the case of the dividing synkaryon formed in conjugation. Further differences from the nuclear phenomena of conjugation are also shown in the formation of the necrochromidium. Thus, the filaments produced from the macronucleus are always short and lie, like the vesicles to which they eventually give rise, along the original traverse of the unfragmented macronucleus. The behaviour of the larger organism was not traced beyond the stage represented by Text-fig. 6B. This circumstance may, of course, be owing to a lack of material. On the other hand, it is quite possible that in the later stages the distinguishing morphological feature of the process is obscured by the fusion of the larger and smaller organisms. On the assumption that three of the four nuclei which are formed by the division of the micronucleus in the larger organism become macronuclear Anlagen, the product of the fusion of the larger and smaller organisms would then be indistinguishable from a post-conjugation distributive form with three macronuclear Anlagen.

It is clear from the above that the process under consideration in this section must be sharply distinguished from normal conjugation. From the information available it may be described as an unusual, endomictic reorganization of the nuclear apparatus.

An Anomalous Process of Micronuclear Reduction

At various periods specimens of *L. tattersalli* are found with minute buds attached to the surface of the left oral quadrant, opposite to the left extremity of the macronucleus (Text-fig. 6C). These buds consist of a small mass of protoplasm surrounding a rounded inclusion which resembles a normal micronucleus in size, form, and reaction (to Feulgen and other nuclear stains). This inclusion is undoubtedly a nucleus which is produced by the fission of the micronucleus of the larger organism, to which the bud is attached. This conclusion is based on the discovery of the simple intermediary stages in bud formation. In the first place the parental micronucleus leaves its bay on the right-hand side of the macronucleus, moves along the anterior border of the latter, and eventually reaches a position below the protoplasmic surface in the area where the bud is later formed. The micronucleus divides into two at this point. One of the daughter nuclei, with a small quantity of undifferentiated protoplasm, forms the bud. Whether the latter is formed by true budding—i.e. by an outflow of the protoplasmic surface—or by fission has not been ascertained. The macronucleus remains unmodified throughout the process.

Buds of a similar type, and up to seven in number in a single lorica, were observed by Ubisch (1913) in *L. platei*. Ubisch regarded bud-formation as a process of micronuclear reduction which preceded conjugation. The evidence for this conclusion seems negligible, and to be based on specimens in which the buds occur, in the same lorica, together with a larger vegetative organism which possesses a fragmented macronucleus. In the larger organism,

however, the figures (ibid.) show clearly that the pattern of the nuclear material resembles that of a distributive generation in *L. tattersalli*. It seems likely, therefore, that Ubisch was led into error through failing to recognize the distributive divisions of the synconjugant.

In *L. tattersalli* the buds are never found in association with conjugants or in any constant relationship to a distinctive phase of the life-history. At present the point of importance appears to be that these buds are actually formed by the organism to which they are attached, and that they are not foreign bodies or degenerated microconjugants. The recognition of this fact has not previously been possible owing to the lack of observations on the intermediary stages of bud-formation.

Summary

1. A study of the bionomics of *L. tattersalli* shows that the initial infection of the host is by the passage of swarmers from the gill-plates of the female host to those of the embryo in the brood-pouch. The transmission of the infection from instar to instar is about 50–70 per cent. effective. It is brought about by (i) the mobilization of the entire colony at ecdysis by an obligatory type of division (second-type division), and (ii) by the penetration of the swarmers to the surface of the new instar through splits which appear in the old cuticle as it is shed.

2. The term 'protoconjugant' is applied to the organism from which the two microconjugants are formed by binary fission. The protoconjugant is formed by the unequal fission of the parent, and not by budding as in *L. platei* (Ubisch, 1913). The divisions which produce the proto- and micro-conjugants are compared with the normal asexual reproductive process (first-type division, Willis, 1942).

3. The conjugation process is described. In view of the early fusion of both conjugants, the term 'synconjugant' is proposed for the macroconjugant after its fusion with the microconjugant. There are no visible nuclear preparations for conjugation in either conjugant before their attachment. The evidence for a state of 'Conjugationserwartung' (Ubisch, 1913) is criticized and shown to be based, in all probability, on specimens produced by the distributive divisions of the synconjugant.

4. The term 'necrochromidium' is proposed for the mass of vesicles formed by the degeneration of the macronucleus in conjugation and endomixis. Before the formation of the necrochromidium the macronucleus of the macro-conjugant becomes elaborately branched.

5. The distributive divisions of the synconjugant are described for the first time. Resemblance to other vorticellinids is shown during the inter-moult period of the host. At ecdysis, the distribution of the nuclear Anlagen is adapted to the obligatory second-type divisions which are undergone by *L. tattersalli* at this period. In some cases one of the divisions of the synkaryon is cytoclastic.

6. A process of apparent endomixis is described. In this process unequal fission produces a small organism, resembling a protoconjugant which fails to separate, and a larger organism. The micronucleus of the smaller organism remains undivided and soon degenerates. The micronucleus of the larger organism undergoes two successive divisions to form four nuclear Anlagen.

7. An anomalous process of micronuclear division and reduction is described. One of the micronuclei produced by the division passes into a small bud of protoplasm. Later the latter falls off and degenerates.

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The Golgi Material and Mitochondria in the Salivary Glands of the Larva of *Drosophila melanogaster*

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Introduction

THIS paper is the second report on a study of the cytoplasmic element in some of the larval tissues of *Drosophila*. As with the previous study (1947), this one lays emphasis on the morphology, distribution, and behaviour of the cytoplasmic elements in the cells.

Material and Methods

For this study 'wild' flies of *Drosophila melanogaster* were used. The cultural conditions under which the larvae were raised were the same as those under which material was obtained for the previous study. Preliminary observations made on some slides convinced me that in order to understand some of the phenomena observed, it would be necessary to study glands from larvae of many different stages of development. Material was therefore prepared with each one of the many fixatives and stains employed in this study from larvae of about 2, 3, 4, and 5 mm. long, and also from those which had already become quiescent for 3 or 4 hours. These, of course, are arbitrary stages and do not have any developmental basis; but as a means of securing material involving a time factor the method has proved of service for my purpose. Fixatives and stains used were the standard ones for demonstrating Golgi material and mitochondria, such as Champy–Kull, Kolatchev, Mann–Kopsch, Benda as modified by Baker, &c. The last-mentioned technique has given me very satisfactory slides for mitochondria, while Mann–Kopsch slides have proved to be most useful for Golgi material observations. Mann–Kopsch material counter-stained according to Altmann, when once the correct duration of the baths in potassium permanganate and sulphurous acid was found, has given me brilliant differential staining of both categories of elements in the same cell.

From the standpoint of structure and normal development, the salivary glands of *Drosophila* larvae have been investigated by Makino (1938), Ross (1939), Sonnenblick (1940), and Bodenstein (1943). This report deals only with the two cytoplasmic constituents in the cells of the glands at various stages of development.

Observations

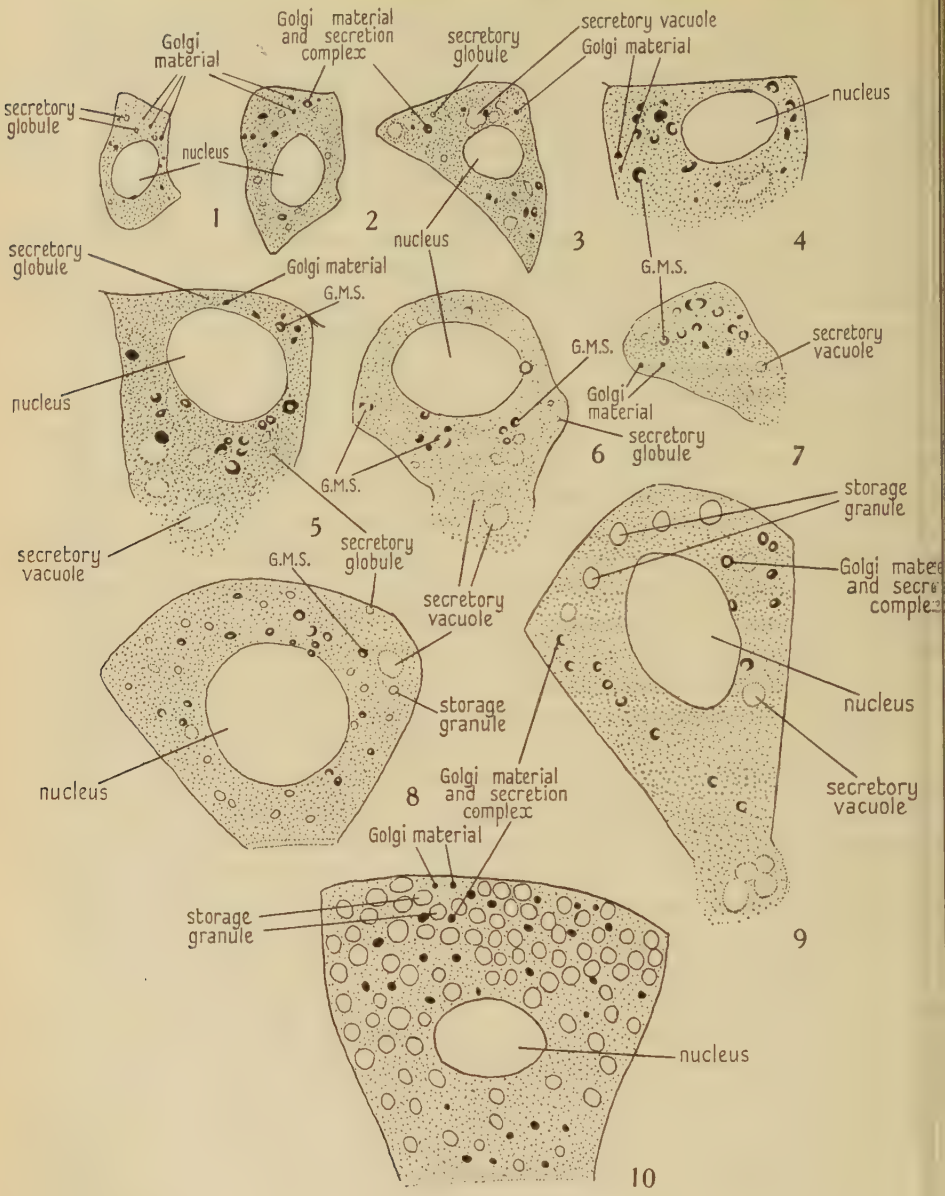
Before presenting my observations it will be helpful to give as a background some facts about *Drosophila* larva salivary glands discovered by the last two investigators mentioned above. Sonnenblick (1940) showed that the increase in size of the glands is due to the growth of the component cells in the plates which initially invaginate to form the glands; the number of these cells remains constant throughout the development of the glands. Bodenstein (1943) in his study of the normal development of the salivary glands reported the following facts which are of interest in connexion with this report: (1) up to the second moult the growth-rate of all the cells is apparently uniform throughout the gland; (2) from the second moult on, however, the cells in a gland do not all grow at the same rate, those in the distal portion of the gland exhibiting a higher rate of growth and consequently being bigger than the more proximally located ones; (3) though at certain stages of development the difference in size between the cells at the proximal end and those at the distal end is quite pronounced, the increase in diameter of a gland in the proximal-distal direction is a gradual one. At no time in the larval life of a *Drosophila* do its salivary glands show a sharp separation into a proximal and a distal portion. 'The form and size of the salivary gland seem thus to be determined by two different growth rates in two different directions: one correlated with age affects the gland as a whole, and the other constitutes a proximal-distal gradient which determines the size of the cells throughout the length of the gland' (Bodenstein, 1943).

My observations, though made with other aims in mind, confirm the facts listed above except the point concerning the number of cells with which a *Drosophila* larva salivary gland begins and ends its development. The nature of my material does not lend itself readily to such a study. This list of facts, so far as they concern this report, may be summed up as follows: the cells throughout a gland from a larva before the second moult are practically all in the same stage of development. This is also true of practically all the cells in a gland of a fully developed larva. However, the cells in the glands of larvae at any stage of development between these two differ among themselves in their degree of maturity according to their location on the long axis of the gland, the more distally situated cells being bigger and more mature. Cells of one stage of maturity found at one locus in the gland of a larva at a certain stage of development are also found at a more proximal position in the gland of a larva more advanced in age. This having been ascertained for a fact, I have found it both simple and instructive, while making my observations, to concentrate on following the changes which the cytoplasmic constituents undergo in cells of different degrees of maturity rather than paying too much attention to the particular stage of development of a gland as a whole. The matter is thus reduced to placing in order the changes observed in the Golgi material and mitochondria as they occur in cells from very young ones to those which have begun to exhibit cytolysis.

Golgi material. In the cells of the youngest material I have prepared, the Golgi material occurs in discrete particles, mostly of an irregular shape, though some may appear roundish, but never of a smooth outline such as chondriomites assume (Text-fig. I. 1). At this stage they appear homogeneous, but occasionally in one or two of them a light area is faintly observable. The number of individual elements in each cell as seen in sections of 5 μ is around twelve. Their distribution in the cell is apparently a haphazard one: they do not seem to favour any particular location in the cell, such as the neighbourhood of the nucleus, the basal pole or the lumen pole region, &c. Besides the Golgi elements very minute droplets or vacuoles of about the same size as the Golgi elements can be seen lying free in the cytoplasm. Since they are not stained and are very small they are not at all conspicuous; but they are unmistakably visible once an observer is made aware of their presence.

In older cells such as depicted in Text-fig. I. 2 and 3 the Golgi bodies have increased in size accompanying a size increase in the cells themselves, though their number seems to have remained about the same as in the cells of the previous stage. The lighter area in some Golgi elements is now easily visible, and the droplets lying free in the cytoplasm are also bigger than those found in the younger cells. The presence of a number of relatively larger vacuoles besides the usual small droplets in the cell as shown in Text-fig. I. 3 is interesting in connexion with what Baptist (1941) discovered in the salivary glands of Hemiptera-Heteroptera. The minute droplets lying free in the cytoplasm do not seem to have a nuclear origin so far as observable evidences indicate. Neither do slides made to demonstrate mitochondria point to a mitochondrial origin for them. Having worked on the Golgi material in *Drosophila* larvae for some time, one is tempted to regard these small spherical droplets as products elaborated by the Golgi elements and set free in the cytoplasm, the bigger vacuoles being simply the result of fusion of two or more of the minute droplets. This idea is amply borne out in cells of the next stages of development (Text-fig. I. 4, 5, 6, and 7). These cells are larger and the individual Golgi bodies have also increased in size. They are thus much more favourable material for critical study. The condition found in cells in these stages as regards the Golgi bodies is such as to duplicate that found in the actively secreting cells of the glandular portion of the proventriculus of the larva (1947). Text-fig. I. 6 shows a cell in which two or three of the secretion droplets are about to be entirely liberated from their respective Golgi shell. The only difference between the two cases is that in the proventriculus the droplets released from the Golgi material do not fuse to form bigger vacuoles as they do in the present case.

All the four figures referred to above show the cells with their lumen ends broken, releasing into the lumen a portion of their cytoplasm together with the secretion vacuoles and droplets. So, it seems, there is on the part of the cells a periodic release of the digestive enzyme or enzymes elaborated by the Golgi bodies. But the cell itself is never wholly destroyed. On the basis of the absence of mitosis and of replacement cells, and of the fact that no



TEXT-FIG. I

All figures are camera-lucida drawings made at ×1,150 the originals, except Text-fig. IV. 26, which is ×510. G.M.S.=Golgi material and secretion complex.

1. A very young cell showing homogeneous pieces of Golgi material in one of which a light centre is barely visible; secretory globules of about the same size as the larger Golgi elements are seen lying free in the cytoplasm. Mann–Kopsch.

2. An older cell than the one shown in Fig. 1; Golgi elements have increased in size and the secretory globules contained within them are more easily discernible; free secretory globules have also increased in size and number. Mann–Kopsch.

nucleus of any cell has been seen to be affected in the process of releasing by the cell of its secretory material, it must be concluded that the cells have the power to repair themselves after the function of discharging the secretion into the lumen has been fulfilled. In this connexion it is interesting to note that cells with a broken inner wall and gland lumens partially filled with secretion are more often observed in younger glands (Text-fig. II. 14) than in those having reached a certain stage of maturity. I shall, however, return to this point in a later paragraph.

In the next stage of growth a cell shows its Golgi elements in just about the same condition (Text-fig. I. 8). But besides the Golgi bodies and their released droplets, both coalesced and uncoalesced, granules of a new type are to be found. These granules have at this time about the same size as the Golgi bodies, but appear to possess a less watery consistency than the digestive droplets. In material osmicated correctly for critical Golgi material study they appear faintly grey. A cell of the next advanced stage shows these granules bigger and more numerous. In such a cell the Golgi bodies present a picture not much different from what can be seen in cells one stage younger. Text-fig. I. 9 shows a few large granules besides the Golgi bodies and their released secretion. This figure is interesting because some of its fused drops of digestive secretion are going through further coalescence in the mass of cytoplasm which has already broken into the lumen of the gland.

From this stage on and so far as the Golgi material is concerned the condition remains about the same, except that as the cells grow bigger it becomes increasingly difficult to observe the droplets and vacuoles. But Text-figs. I. 10, and II. 11 and 12 show the sequence of events in the development of the granules, leading eventually to a stage wherein a cell may be said to be literally filled with them. These figures represent cells in which the Golgi bodies are a little over-impregnated, as a result of which the drop of secretion contained in each Golgi element does not stand out so clearly. It will also be noticed in Text-fig. I. 10 that there are more of these granules at the basal end of the cell—a condition very often observed. This seems to suggest that the origin and growth of these granules begin from the basal pole of the cell. Text-fig. II. 12 shows a cell with its full load of granules already developed. In fact, it is evident that its lumen pole has begun to show signs of disintegration as indicated by a layer of very darkly stained cytoplasm.

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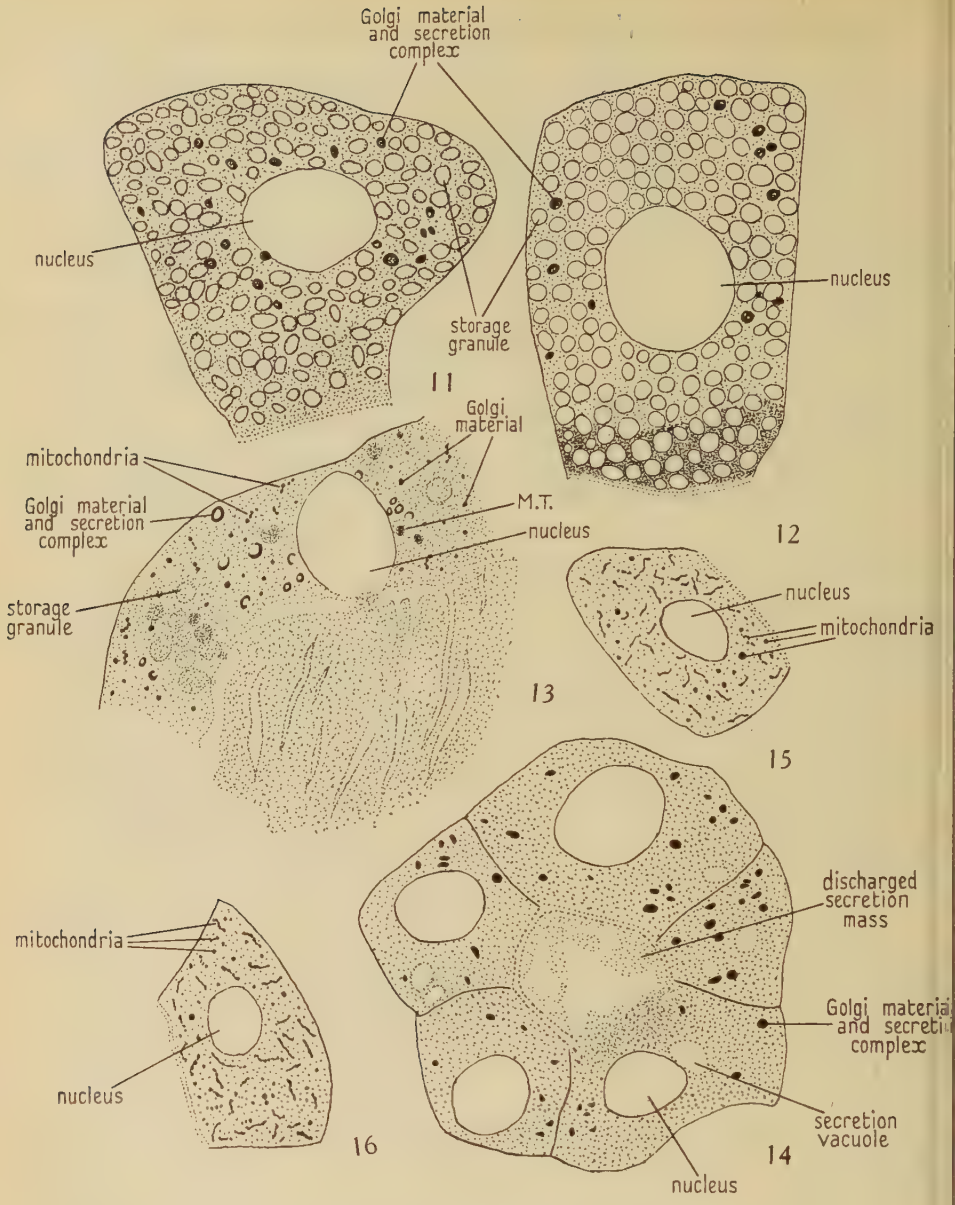
3. A still older cell in which vacuoles of secretion are formed as a result of fusion of two or more individual secretory globules. Mann–Kopsch.

4–7. Four cells of increasing degree of maturity showing changes undergone by Golgi elements and their spatial relation to the secretory globules. Fig. 6 illustrates the setting free of the secretion droplets as a result of the breaking of their respective confining Golgi shell. The cells are depicted with their lumen end broken, releasing into the gland lumen vacuoles of secretion and a part of cytoplasm. Mann–Kopsch.

8–9. Two cells of different stages of development showing the appearance of the storage granules in the cytoplasm. Mann–Kopsch.

10. A cell of advanced maturity showing the storage granules increased in size and number. Kolatchev.



TEXT-FIG. II

11–12. Two mature cells full of storage granules. Fig. 12 shows the cell with its lumen end exhibiting signs of cytolysis. Kolatchev.

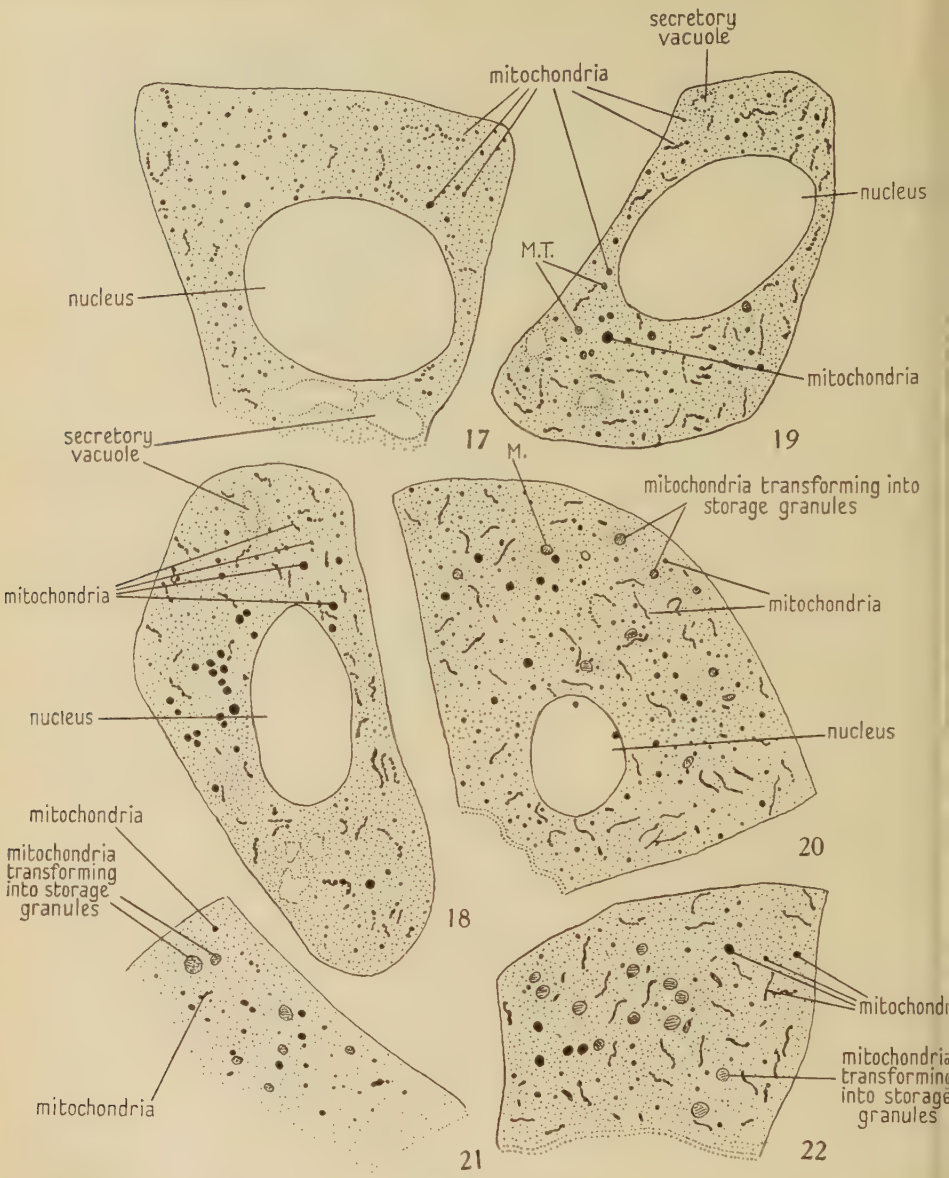
13. A portion of a cross-section of a salivary gland from a quiescent larva showing the lumen space filled with disintegrated cytoplasm; part of the epithelial cell or cells are still recognizable, in which are seen the nucleus, Golgi-material-secretion complexes, mitochondria, and storage granules. Mann–Kopsch–Altmann. M.T. = mitochondria.

14. A cross-section through a young salivary gland showing one of the cells releasing secretory material and part of its cytoplasm into the lumen which is partially filled with the discharge. Kolatchev.

15–16. Two young cells showing the condition of mitochondria in such cells. Benda–Baker.

During cytolysis, when the larva has become quiescent, or even a few hours before that, the cell and the material of disintegration lying in the lumen look somewhat as shown in Text-fig. II. 13. It shows a cell in which cytolysis has already touched the inner tip of its nucleus. The mass of disintegrated material from the glandular cells practically fills the whole lumen. The clefts in the mass of disintegrated cytoplasm are probably due to the technique to which the material had been subjected. In what is left of the cell Golgi bodies are rather numerous and do not show any sign of disintegration. Chondriomites are also numerous and they no longer aline themselves into threads so often as in younger cells. There are also granules, of course, and they stain in various shades of colour. Roughly, their ability to stain decreases as they become bigger. It is interesting to observe that once nuclear material, cytoplasm, and cytoplasmic inclusions are in the lumen of the gland, they soon lose not only their morphological identities but also their individual stainability which they possessed when still within the cell.

Mitochondria. Mitochondria appear in the form of both chondrioconts and chondriomites in the salivary gland-cells of all stages of development. They are numerous and are distributed evenly throughout the cell. In very young cells the chondrioconts of a granular construction seem to be more numerous than the other form. The chondriomites are not uniform in size. Some of them have a diameter of about the thickness of the chondrioconts, but there are others which are considerably bigger (Text-fig. II. 15 and 16). Vacuoles and droplets are also seen in mitochondria slides, although no evidence could be observed which would incline one to attribute a mitochondrial origin to them. In a more advanced stage the cells show more and bigger chondriomites. Some of the bigger ones appear to have a diameter four to five times the thickness of the filamentous form. The small chondriomites are also present and the two extremes are connected by a graded series (Text-fig. III. 17 and 18). Text-fig. III. 19 shows an interesting phenomenon which, up to this stage of development, is yet new. There appear among the bigger chondriomites some which do not stain so deeply as the rest. These lighter-coloured ones reach a rather big size as the cell advances towards full development, and it seems also there is a rough parallelism between the size and the ability to stain of these transforming chondriomites: power to stain lessens as their size increases. This situation is best brought out by viewing Text-fig. III. 17–22 in rapid succession. And Text-fig. IV. 23 and 24 represent two cells in which the enlarged and lighter-coloured chondriomites, if they can still be so called, grade imperceptibly into the fully formed granules which take only a faint trace of colour, if any at all. These are the granules which will eventually fill up the cell, giving the characteristic cytological appearance to the cells of glands of a fully grown larva regardless of whether the material has passed through fixatives suitable for mitochondria, for Golgi material, or for the nucleus and the respective subsequent treatments. Indeed, it is difficult for anybody who has seen such cells to attribute the origin of these granules to any organelle in the cell other than mitochondria.



TEXT-FIG. III

17. A fairly young cell in which some of the chondriomites have attained a diameter four to five times that of the small ones. Note the two big vacuoles of secretion near the broken lumen end of the cell. Benda–Baker.

18. A cell in which some of the chondriomites have attained even greater size, though all of them still stain as intensely as the smaller ones. Benda–Baker.

19. A cell showing the enlarged chondriomites no longer taking mitochondrial stain as intensely as the smaller ones. Benda–Baker. M.T. = mitochondria.

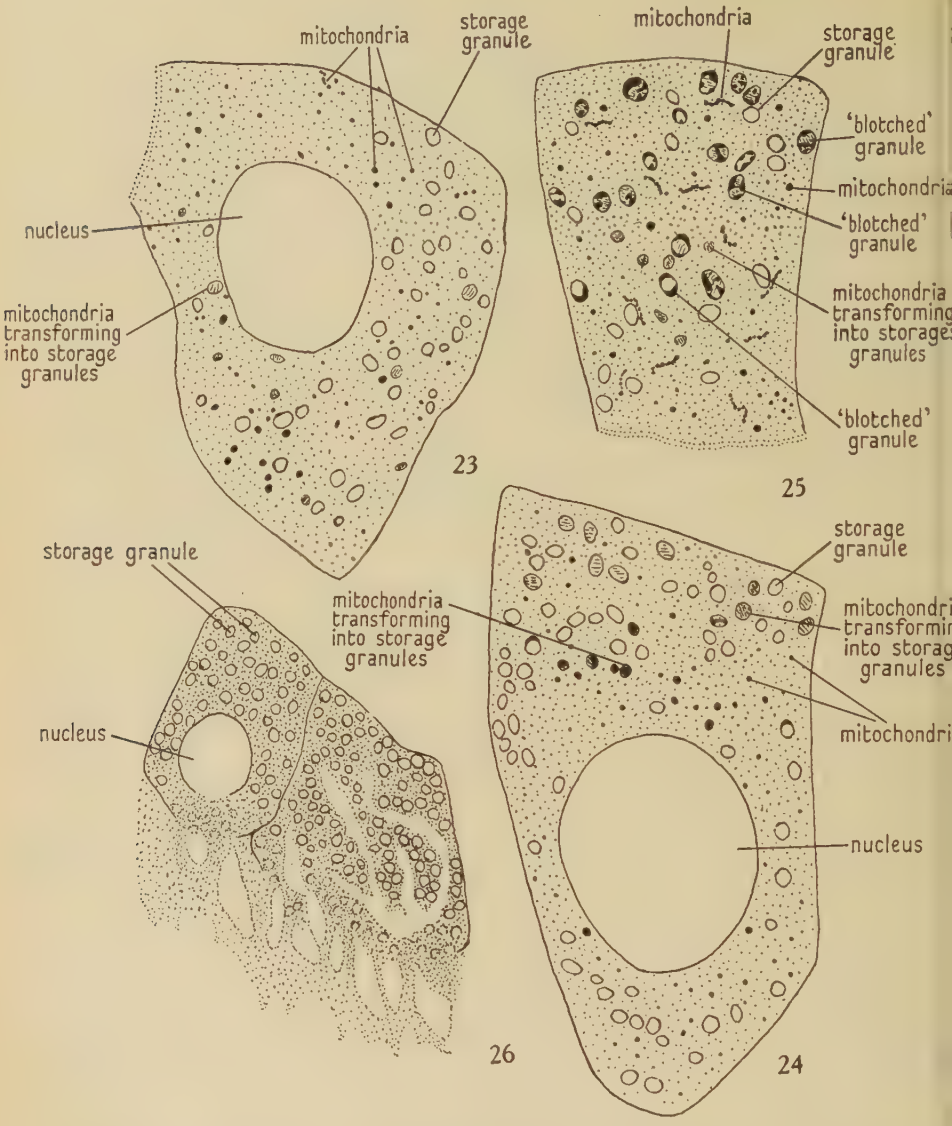
20–2. Three cells in which the light-staining or transforming chondriomites have become more numerous. Benda–Baker. M = mitochondria transforming into granules.

Text-fig. IV. 25 shows a somewhat puzzling phenomenon. Rather frequently some granules may be encountered in a cell, usually in one filled, nearly or completely, with storage granules, which show splashes of intensely blue or faintly red colour according to the stain applied. Some may exhibit a coloured crescent capping partly around them. This phenomenon is observed only in cells one or two steps earlier than the stage of cytolysis. It must be regarded as one stage in the transformation from mitochondria to the mature granules, since, in cells having already begun to undergo cytolysis, blotched granules have never been seen (Text-fig. IV. 26). It is difficult to explain such granules unless one assumes that there are two methods for the chondriomites to transform themselves into granules. One is a uniform transformation throughout the granules; and the other an uneven one, leaving, before the process is completed, certain sections in the grain still of the chemical composition of mitochondria. Even so, however, it would still be difficult to explain the fact that the blotches on the granules often seem to stain more intensely than the tiniest chondriomites found in the same cell. Whatever the true nature of such blotches and crescents may be, the evidence pointing to a chondriomite origin for the granules is nearly irrefutable.

Discussion

In the salivary glands of the larva of *Chironomus*, neither Parat and Painlevé (1924), nor Beams and Goldsmith (1930), nor Gatenby (1932) saw any evidence that either Golgi bodies or mitochondria play a part in secretion synthesis. Beams and King (1932) came to the same conclusion in the salivary glands of the grasshoppers. Beams and Wu (1929), in their studies on the spinning glands of *Platyphylax designatus*, had the following to say: 'Throughout the activity of the gland, the Golgi apparatus changes little from normal condition, both in form and distribution. No relation of Golgi apparatus to the secretory phenomenon is apparent.' Wu (1930) reported that in the spinning glands of the larva of *Galleria mellonella* the secretory substance has a nuclear origin and that mitochondria play a negligible or at most a minor role. He could not detect any sign that Golgi bodies play a part in secretory synthesis. Lesperon (1937) also disclaimed any role by the two cytoplasmic elements in the synthesis of secretion in the silk glands of silk-worm and other insects. Baptist (1941) studied the salivary glands of an extensive list of Hemiptera-Heteroptera. He found in *Notonecta*, for instance, that the cells have developed the mechanism of forming zymogen-granules and are thus capable of producing a large quantity of secretion at short notice. To store this, the cells have also developed collecting vacuoles. But he also said that although both Golgi bodies and mitochondria typical of insect tissue are present in the cells, they 'bear no special relation to the nucleus or secretion granules'. He published, however, no figures of either Golgi material or mitochondria as seen in his material.

The result of my study on the salivary glands of *Drosophila* larvae, however, very clearly indicates that the secretory droplets are elaborated in the Golgi



TEXT-FIG. IV

23–4. Two cells showing transition from late stages of transforming chondriomites to slightly coloured or colourless storage granules. In Fig. 24 all the stages of transition from intensely stained minute chondriomites to colourless storage granules are shown. Benda–Baker.

25. A cell containing 'blotched' storage granules. Benda–Baker.

26. Two cells undergoing cytolysis, their lumen ends broken and their disintegrated cytoplasm lying in the gland lumen. Only storage granules are shown in the cytoplasm. Kolatchev.

bodies. Briefly, the individual Golgi bodies are small roundish or irregularly shaped bits, and as each increases in size a lighter area (secretory droplet) appears in it, forming what I called in my previous study (1947) a Golgi-material-and-secretion complex. When a complex has reached a certain size the drop of secretion is released into the cytoplasm, and, as a result of the fusion of two or more of them, larger vacuoles are formed and stored in the cell. The Golgi-material shell now has broken into several small pieces, each of which presumably is potentially capable of elaborating another droplet of secretion. It may be said that so far as the Golgi material is concerned, its morphology and behaviour, in relation to the elaboration of secretion as observed in the cells of the salivary glands, almost exactly duplicate what has been seen in the cells of the glandular portion of the proventriculus (Hsu, 1947). I have seen no indication whatever that the secretory material is first separated under the influence of the mitochondria and then moved up to the Golgi material to be matured into secretory droplets. Neither have I seen anything which may suggest a nuclear or nucleolar origin for them. The only difference in the two cases is that in the proventriculus cells fusion of smaller droplets into bigger ones was not observed. This, however, is really a point of difference between the two types of cells in their methods of discharging their secretory material and does not concern the question of the origin of secretion.

With regard to the structure of Golgi bodies, I can only restate briefly what I have already said in my other paper (1947). My observations would only allow me to recognize the existence of a homogeneous substance, which I prefer to call Golgi material. This I believe corresponds to the 'Praesubstanz' of Hirsch (1932), the dense lipoid-containing substance of Baker (1944), and the osmiophilic or agentophilic substance of others. When a light area appears in a piece of Golgi material I have so far simply regarded it on observable evidences as a drop or granule of secretion viewed through a film of Golgi material, and have preferred to call the whole by the clumsy name 'Golgi-material-and-secretion complex'. It may be noted here that in my paper dealing with the midgut epithelium of *Drosophila* larvae (1947) I compared the structure of a 'complex' to a Golgi-material bowl in the hollow of which is set a drop or globule of secretion. But in spite of the lack of a visible film over the apparently exposed area of the secretion in some cases, my study since then has given me confidence to regard the structure of all the complexes at all stages as a vesicle. I have seen nothing which would make it necessary for me to differentiate a piece of Golgi material into a chromophilic and a chromophobic component at any stage of its activity.

In cells crowded full with granules the cytoplasm is squeezed into very narrow films whose widths are clearly indicated by the presence in them of chondriomites and chondrioconts. These elements are seen deposited around the granules; and in sections in which the granules are not sufficiently clearly coloured, it is by means of the distribution of the chondriome that the boundary of the individual granules is made out. It appears reasonable to say that this paucity of cytoplasm in the cell toward the end of the life of a

larva is at least one of the contributing factors why in cells packed full with granules hardly any droplets or vacuoles (indicating elaboration of digestive secretion) or broken lumen end (indicating discharging of secretion) could be observed.

This, however, is not the case in younger cells in which the granules either have not yet made their appearance or have not yet become so numerous and big as to occupy most of the space within the cell. In such cells there are observed more free droplets and vacuoles and more Golgi bodies containing secretion. Cells with broken inner wall and gland lumens filled with discharged secretion are much more often encountered in young glands or that section of older ones where comparatively younger cells are found. The larval life of *Drosophila*, except when moulting, may be said to be a period of ceaseless feeding. The cells of a salivary gland, up to a certain size, appear to go through a continual process of elaborating digestive fluid and storing it in vacuoles, ready to discharge it by means of a merocrine mechanism into the gland lumen whenever conditions demand. There does not seem to exist in them a clear-cut secretory cycle as has been observed both in the cells of the glandular portion of the proventriculus and the anterior portion of the midgut (Hsu, 1947).

As to the granules which give to the mature cells their characteristic and striking appearance in histological preparations, it appears that to secrete digestive enzymes is but one of the functions of the salivary glands in a *Drosophila* larva. When the cells have grown to a certain size another function, that of storage, begins to go on simultaneously with the function of secretion. Storage material in the form of granules begins to be transformed from individual chondriomites. I regard these granules as storage food, and not as having anything to do with digestion, on the basis of the following observations: (1) they have never been seen discharged into the gland lumen during the whole feeding period of the life of a larva; (2) they are seen to disintegrate into a homogeneous mass and to be set free into the lumen space only some time after the larva has ceased feeding; (3) unless the cell is filled with them, they are always seen accumulated at the basal end of the cell, while the secretion droplets and vacuoles have often been observed at the lumen end.

Regarding the storage granules found in older salivary gland cells, it is worth while to note that Painter (1945), in his interesting work on secretion with reference to the origin of cytoplasmic nucleic acid, apparently took these granules to be globules of digestive secretion. In describing his Fig. 17 he said it 'is filled with clear alveoli which are interpreted as secretory product which have not yet passed out of the cell'. But his Figs. 17 and 20 present to me the very familiar appearance of cells filled with what I now have evidence to regard as storage granules. His Fig. 21 is also convincingly that of a cell undergoing cytolysis, and does not, as he apparently then thought, represent a mere discharging of the 'secretory globules'. I have never seen these granules discharged into the lumen of the gland unless as a result of cytolysis

when the larva is about to pupate. The larva gland cells do not 'secrete' these granules and then empty them into the gland lumen and begin to elaborate another batch and so on. The formation and accumulation of these granules proceed as a linear process and are not carried on in the cell in cycles. They visibly begin in the fairly young cells and stop presumably after the larva has ceased feeding. I have seen histolyzing glands from larvae which have stopped feeding but are still very actively crawling about before becoming quiescent. It would seem that the stored food is drawn upon almost immediately after food intake is stopped. Salivary glands are apparently the first organs to cease functioning in metamorphosis, since as organs they no longer answer any need of the organism at the time. It would be interesting, therefore, to re-examine the nucleic acid situation, correlating the amount and distribution of it not with the larger granules found in the older cells but with the smaller droplets and vacuoles found in the younger cells.

Ross (1939), in her developmental study of the salivary glands in the larva of *Drosophila melanogaster*, also misinterpreted the large granules to be globules of digestive secretion, though she reported that the salivary gland cells of adult *Drosophila* show no such 'globules'.

Summary

1. The salivary glands in the larvae of *Drosophila* show evidence of serving two functions: (1) production of digestive secretion, (2) accumulation of reserve food for the period of pupation. The two functions proceed simultaneously within the same cell during certain stages of its development.

2. A single droplet of digestive material has been seen to originate and grow within each Golgi body in the gland-cells. When a certain size is reached the droplet is released into the cytoplasm and by the fusion of two or more of them bigger vacuoles are formed. The secretory material is discharged into the lumen by means of a merocrine mechanism. Neither mitochondria nor nucleus has been observed to take any visible part in the elaboration of secretion droplets.

3. The storage granules found in older and larger cells have been observed to be direct transformations of chondriomites, and neither the Golgi material nor the nucleus shows any sign of participation in the formation of these granules.

4. From the standpoint of morphology and behaviour, the Golgi bodies found in the salivary gland cells are the same as found in the cells of the glandular portion of the proventriculus and the epithelium of the anterior portion of the midgut of the larva.

5. My observations do not lend themselves convincingly to a two-component conception of the structure of Golgi bodies.

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Phosphatase Activity of *Drosophila* Salivary Glands

BY

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THE presence of alkaline phosphatase in chromosomes has been demonstrated by means of histochemical staining methods (Danielli, 1946; Krugelis, 1942, 1946). These studies indicated the effects of various fixatives and substrates on the subsequent staining reactions. The present study presents certain quantitative aspects of the effects of acetone, alcohol, and pH on the phosphatase activity of whole salivary glands of *Drosophila*.

Material and Methods

Paranitrophenyl phosphate (Bessey, Lowry, and Brock, 1946) was used as a substrate. Magnesium was omitted after failure to detect increased activity with added magnesium. Hydrolysis was proportional to enzyme concentration under the conditions employed. In order to reduce the rate of inactivation of the enzyme the determinations of alkaline phosphatase activity were made at pH 8·6 rather than at the more alkaline optimum of maximal activity.

To determine the general characteristics of the phosphatases present in this material, several dozen glands were pooled and attempts made to get reproducible extracts and suspensions. This method was unsatisfactory. The salivary glands vary greatly in size and enzyme activity from one individual to another; but the single glands of a pair appear to be identical in size and enzyme content. Accordingly it is possible to control factors such as differential extraction of the enzymes and individual variation as follows: provided that enzyme blanks (the paranitrophenol colour value of samples without substrate) are negligible, as is the case here, each larva provides two aliquots. A pH curve or other comparison can then be run as indicated in Table I, where letters represent larvae and postscripts the glands of the pair.

Table I

pH	4·6	5·0	5·4	5·8	&c.
Samples	A_1	A_2			
		B_1	B_2		
			C_1	C_2	
				D_1	&c.
	K_1		K_2		
		L_1		L_2	

Individual glands were dissected in saline and fixed in acetone in tubes 6×25 mm. The acetone was replaced three times during the first 10 minutes; at the end of the fixation period all but 1·0 c.mm. of acetone was removed and 50 c.mm. of buffer added (M/25 diaminobutane pH 7·3–10·2, or barbital pH 3·0–7·3). After a suitable extraction period 50 c.mm. of substrate (adjusted to the pH of the extraction buffer) were added. The preparations were incubated 1–3 hours at 30° C., depending on size of gland. The gland was removed with a glass needle and the reaction stopped by addition of 15 c.mm. of 1·0 N. NaOH. The optical density of 100 c.mm. samples was measured on a Beckman spectrophotometer using Lowry micro-cells. Enzyme blanks were run on some pairs in each series but their colour was invariably negligible. The glands remained intact through several consecutive determinations.

Observations

Fresh extracts. When fresh glands were placed in distilled water or buffer for varying periods prior to addition of the substrate very little enzyme activity was found. In one case 20 glands were placed in 100 c.mm. of distilled water and subjected to the gentle pounding of an electromagnetic stirring bead for 2 hours. In this instance less than 10 per cent. of the enzymatic activity of the acetone-fixed aliquot was found. In preliminary experiments it was found that whenever, during dissection, a cell of a gland was penetrated by the dissecting needle, the subsequently determined activity of the gland was higher than that of the uninjured gland (cf. Table IV, B3 and B4). It is evident that either the cell membrane or more probably the membranous capsule of the gland effectively prevents extraction of the enzyme and entrance of the substrate even in unphysiological media.

This fairly obvious property of membranes has occasionally been measured (Linderstrøm-Lang and Holter, 1933, and Doyle, 1938). When the gland is frozen (Table II, B1, B2; C1, C2) or is fixed (Table II, Q1, Q2; R1, R2) this property of the gland surface is diminished.

Table II. *Phosphatase Activities of Individual Salivary Glands*

Gland	*Treatment*	*pH*	*Activity*
B1	Fresh	8·6	0·90
B2	Frozen	8·6	1·37
C1	Fresh	8·6	0·160
C2	Frozen	8·6	0·400
Q1	Fresh	8·6	0·047
Q2	Acetone 20 min.	8·6	0·321
R1	Fresh	8·6	0·056
R2	Acetone 24 hrs.	8·6	0·400
S1	Acetone 24 hrs.	9·5	0·700
S2	Acetone 24 hrs.	8·6	0·472
Fat	Acetone 24 hrs.	8·6	0·035

Fat is found closely applied to the glands and it is sometimes difficult to remove all of the fat without injuring the glands. That small traces of fat contain negligible quantities of phosphatase is shown in Table II, where pieces of fat twice the volume of the glands of specimen S were assayed along with the glands.

Fixed glands. Several pairs of glands were fixed in acetone for 24 hours and allowed to stand in distilled water for 15 minutes. Negligible activity was found in the extract and the activity of the extracted gland corresponded with the activity in glands not extracted. When extracted for 24 hours at 5° C. in M/25 diaminobutane buffer pH 9·5, about one-third of the total activity was found in the extract. In another series glands fixed in acetone for 9 days and extracted for 16 hours at 5° C. with M/50 barbital pH 7·3 gave 20 per cent. of the total activity in the extract. In this series redetermination of the residual phosphatase activity gave 87 per cent. of the initial residual activity. The alternate glands of pairs treated at the same pH with diaminobutane buffer gave corresponding results.

The total enzymatic activity of glands fixed in acetone is higher (at pH 4·4 and 8·6) than that found after similar treatment with 80 per cent. alcohol (Table III). Here 75 per cent. of the pH 8·6 acetone value was found after

TABLE III. *Phosphatase Activities of Glands after Fixation in 80 per cent. Alcohol and in Acetone at pH 8·6*

Gland	*Time of fixation*		*Observed activity*	*pH*	*Notes*
	Alcohol	*Acetone*			
H1	2 hrs.	..	0·408	8·6	= 70 per cent. of H2 (Alk-p-ase).
H2	..	2 hrs.	0·590	8·6	
J1	1½ ,,	..	0·175	8·6	= 75 per cent. of J2.
J2	..	1½ ,,	0·235	8·6	
K1	2½ ,,	22 ,,	0·185	8·6	= 77 per cent. of K2.
K2	0	24 ,,	0·248	8·6	
L3	2½ ,,	22 ,,	0·119	8·6	= 73 per cent. of L4.
L4	0	24 ,,	0·171	8·6	
M7	2½ ,,	22 ,,	0·110	4·4	= 20 per cent. of M8 (Acid p-ase).
M8	0	24 ,,	0·585	4·4	

80 per cent. alcohol treatment and 20 per cent. of the pH 4·4 acetone value. Since the results obtained with specimens J and H might be ascribed to a differential effect of acetone and alcohol on the penetrability of the cellular material, the subsequent specimens (K, L, M) were treated with acetone as well after the alcohol fixation.

Redeterminations on the same gland. Using whole acetone-fixed glands, only a fraction of the enzyme is extracted during the initial determination. A second determination may then be made under similar or altered conditions to examine the effect of the first determination on the residual enzyme.

Representative data from these experiments are given in Table IV. Here it would appear that 60 per cent. of the pH 8·6 activity survives a determination (Detn.) at pH 4·4; that 45 per cent. of the pH 4·4 activity survives a determination at pH 8·6; that 38 per cent. of the pH 8·6 activity survives a determination of pH 8·6; and that 87 per cent. of the pH 7·3 activity survives a determination at pH 7·3. The decreased activity after the more alkaline initial determinations is in keeping with other findings (Lundsteen and Vermehren, 1936) in which the pH optimum is more alkaline for short digestion times than for longer ones.

TABLE IV. *Redetermination of Phosphatase Activities on Glands fixed in Acetone for 24 Hours*

	pH		*Activity*		
Gland	*Detn. 1*	*Detn. 2*	*Detn. 1*	*Detn. 2*	*Notes*
T1	7·3	7·3	0·990	0·870	$\frac{870}{990}$ = 87 per cent. of p-ase after neutral Detn.
A1	4·4	8·6	0·317	0·244	$\frac{244}{413}$ = 60 per cent. of Alk-p-ase after acid Detn.
A2	8·6	4·4	0·413	0·150	$\frac{150}{317}$ = 45 per cent. of Ac-p-ase after alk. Detn.
B3	8·6	8·6	0·351	0·133	B4 punctured with needle. $\frac{133}{351}$ and $\frac{183}{462}$.
B4	8·6	8·6	0·462	0·183	= 38–9 per cent. of Alk-p-ase after alk. Detn.

Gomori preparations for both acid and alkaline phosphatase made subsequently to the initial quantitative determinations showed little differences from those made immediately following the respective fixations.

DISCUSSION

That phosphoesterases from different sources vary with respect to stability to acetone and to alcohol is indicated by comparison of these results with those of Stafford and Atkinson (1948, rat tissues), who found higher alkaline phosphatase activity after alcohol (80 per cent.) than after acetone fixation.

The absence of appropriate values for fresh extracts and homogenates in this study prevents an estimation of the degree of destruction of alkaline and acid phosphatase by the alcohol and acetone. Determination of the ratio of activities found to true enzyme content requires the preparation of suitably cytolysed samples without major losses.

It is obvious, however, that acetone preserves more phosphatase activity in our material than does alcohol.

In specimen B, Table IV, B4 was deliberately punctured during dissection with resulting higher activity. The amount of enzyme lost between deter-

mination 1 and 2 was essentially the same in glands B_4 and B_3 (unpunctured). This would indicate that the increased activity of B_4 over B_3 is due to better access of substrate, and that at pH 8·6 (for 2 hours) the decreased activity in determination 2 as compared to the initial determination is due to inactivation rather than to extraction of the enzyme.

With sectioned rabbit material (Doyle—in the press) there is much greater extraction of enzyme (especially of acid phosphatase) than from these whole glands. Furthermore, acid phosphatases from many sources are much more labile than alkaline phosphatases. Gomori (1946) used acetyl cellulose to reduce losses and translocation of enzyme. A coating on the section which prevents diffusion of the enzyme will to some degree also affect the rate of penetration of the substrate. The problem then arises as to whether, in the absence of completely cytolysed cells in extracts and in the absence of free access of substrate to cell structure in sections, the activities observed under given experimental conditions can represent the enzymatic contents of the material. Only when the true enzymatic contents of cellular structures can be ascertained will there be a better basis for conjecture concerning the role of the enzymes, but the demonstration of maximal enzymatic *content* will not of itself indicate the normal physiological *activity* with respect to that enzyme.

This work was aided in part by a grant from the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

Summary and Conclusions

The phosphatases in the cytoplasm and nuclei of *Drosophila* salivary glands are better preserved by fixation in absolute acetone than in 85 per cent. alcohol. In whole glands there is relatively little extraction of the enzyme during assay. Phosphatase activity is more resistant to incubation at neutrality than at pH 8·6, but in this material there is sufficient residual enzymatic activity to permit redetermination of alkaline, neutral, or acid phosphatase activity by staining methods after an initial quantitative determination. The state of the membranes of the gland affects the penetration of the substrate sufficiently to limit the activities obtained.

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The Accumulation of Carotenoids in the Golgi Apparatus of Neurones of *Helix*, *Planorbis*, and *Limnaea*

BY

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Introduction

THE existence of coloured granules in neurones of pulmonate gastropods is well known through the work of Legendre (1909) and others. The coloured matter has been usually described as 'lipochrome', a term that Lison (1936) regards as bad since it covers two distinct chemical classes (the carotenoids and the chromolipoids) and has been used in different ways by different authors.

Thomas (1948) has recently produced evidence for considering these coloured granules as a Golgi product, a view not in agreement with that regarding the Golgi apparatus advanced by many workers.

This paper presents the results of a histochemical examination of the coloured substance, and a repetition of some of Thomas's work. The coloured granules contain carotenoid and appear to be formed in the interna of the Golgi bodies.

Material and Methods

Thomas worked with the cerebral ganglia of *Helix aspersa*, in which many of the neurones are very large. The amount of coloured matter is usually small even in the largest cells; there is very much more in the neurones of *Limnaea stagnalis* (L.) and *Planorbis corneus* (L.), both very common freshwater snails. In the former the central nervous system is distinctly coloured by the pigment. In the latter the bright red coloration is due to granules inside the cells and to a certain amount of haemoglobin in the blood as well.

The following histochemical methods were employed:

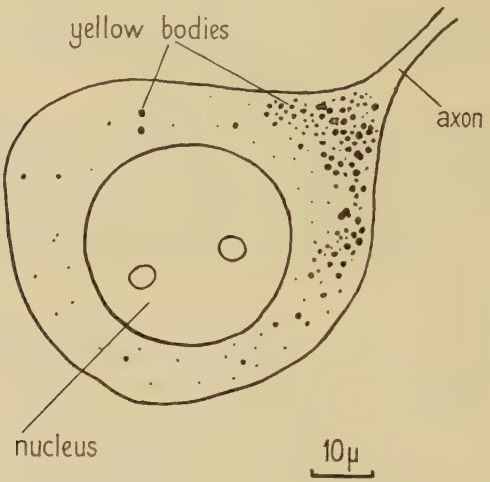
(i) For lipoids in general, material was fixed in formal-calcium, and frozen sections were cut and coloured with sudan black B (Baker, 1944). Sudan black is specific for lipoids (Lison, 1936; see also Cain, 1947*a*). It does not colour pure carotenoids (Lison, 1936, p. 245) and, of course, it does not colour *solid* lipoids. Lison includes the carotenoids under pigments. As they are soluble in lipoid-solvents, they are included here under the heading of lipoids.

(ii) Baker's acid haematein test for phospholipines (Baker, 1946, 1947; Cain, 1947*b*) was used, with pyridine extraction as control.

(iii) For detection of carotenoids the Carr–Price reaction (Carr and Price, 1926) was used. With antimony trichloride ($SbCl_3$) in chloroform carotenoids and vitamin A give a blue coloration which is not permanent.

In addition, the granules were tested with concentrated sulphuric acid, and with iodine (Lison, 1936, p. 245). With these reagents carotenoids give a deep-blue colour.

As a supplementary test, sections were exposed to light and air and the rate of fading was noted.



TEXT-FIG. 1. Diagram of the distribution of bodies visible in a neurone of *Helix*.

Living cells were observed in sodium-calcium saline (Baker, 1944), and were stained supravitally with neutral red chloride, methylene blue (BDH), nile blue, and Janus green B (Höchst).

Mann–Kopsch preparations were made of neurones from all three species, and Thomas's variant of the Mann–Kopsch technique was used with *Planorbis*.

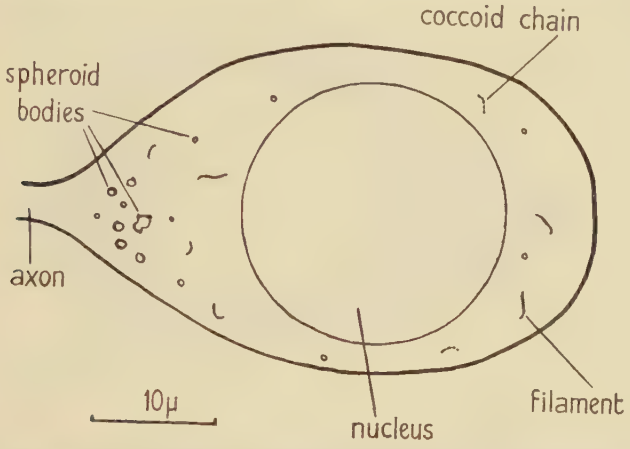
Helix neurones were fixed in Helly, postchromed, stained with Altmann's acid fuchsine, differentiated with sodium carbonate solution, and counterstained with methyl blue (Cain, 1948).

RESULTS

In living neurones of *Helix* a large number of bodies can be seen, which are either subspherical or irregular. These latter are described by Thomas as mulberry forms. The largest are often distinctly yellow in colour. These bodies are scattered throughout the cell, but more and more thickly towards the axon hillock, in which there is usually a large number (Text-fig. 1). On close examination there may be seen a cap or a granule or several granules

adhering to the rim of such bodies. In *Limnaea* and *Planorbis* these bodies are more evident, very numerous, and quite brightly coloured yellow, the largest being a faintly reddish or brownish yellow, and rather more irregular than in *Helix*. Their distribution is the same.

With methylene blue (1 in 10,000) both the bodies and their associated granules or caps are stained, the latter very deeply. A similar effect is produced, but less clearly, with neutral red chloride and with nile blue. Neutral red is the least satisfactory with *Planorbis* and *Limnaea* because in them



TEXT-FIG. 2. Diagram of the relation between the filaments and coccoid chains shown with Janus green, and the spheroid bodies.

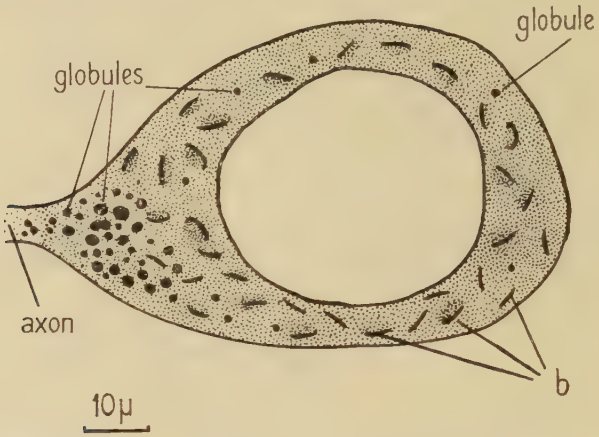
nearly all the bodies are already coloured and the contrast is not great. Methylene blue is very satisfactory.

When neurones were exposed to the vapour of osmium tetroxide, the rims of these bodies, together with their caps and granules, become blackened. Prolonged action results in the blackening of the whole body.

Minute filaments and coccoid chains were not distinctly seen in living cells. On application of Janus green B (Höchst), after many attempts, very distinct filaments were seen in living cells of *Helix* (*Planorbis* and *Limnaea* neurones were not investigated because of the multiplicity of spheroids which make for obscurity). These were very thin and short, sometimes bent or kinked. Coccoid chains were seen most distinctly in a damaged cell; it is possible that they are filaments beginning to break up. In distribution the filaments do not resemble the spheroid bodies, which are also clearly visible, and are not associated with them. They are scattered throughout the cytoplasm and show no tendency to concentration in the axon hillock (Text-fig. 2). They were seen in only a few cells, lying next to those on the outside of the teased-out cell-mass, which were dead and stained diffusely.

Mann–Kopsch preparations were made of neurones of all 3 species, 6 days being found a suitable time of osmication. In preparations from *Helix*

batonnettes as usually described were to be seen in nearly all cells, sometimes with an associated 'archoplasm' but quite often without. Sometimes the cytoplasm contained nothing else, but on occasion there were black or grey spheroidal bodies. These might occur anywhere in the cytoplasm, but where cells were cut through the nucleus and axon hillock it could sometimes be seen that there was an aggregation in the hillock (Text-fig. 3). In preparations of *Limnaea* and *Planorbis* neurones much the same pictures were obtained, but in general the batonnettes were less obvious and the globules more so.



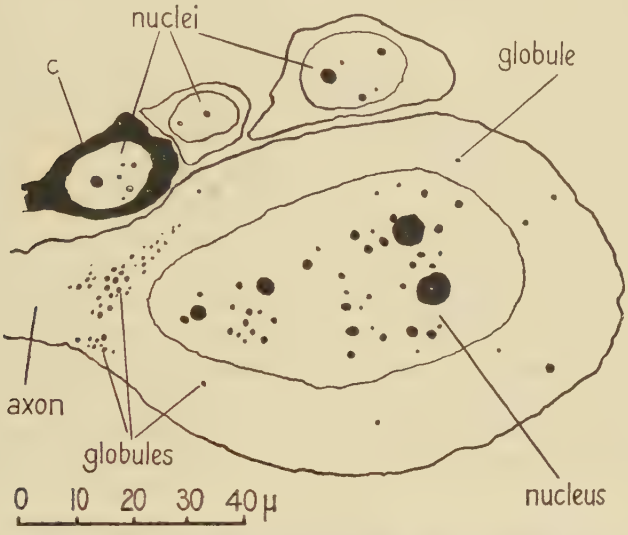
TEXT-FIG. 3. Diagram of the distribution of globules and batonnettes in a Mann–Kopsch preparation. *b*, batonnettes, mostly with archoplasm. Nucleus blank.

In distribution, the globules corresponded exactly to the coloured granules and complexes seen in the living cells. The batonnettes did not correspond to these and, as in *Helix*, were scattered throughout the cytoplasm.

Thomas describes only batonnettes in his Mann–Kopsch preparations, but was able to show globules by bleaching with Veratti's acid permanganate (followed by oxalic acid to remove the brown deposit of oxide) and then colouring with sudan black, when globules appeared in positions quite unrelated to those of the batonnettes which had been shown by osmium. When neurones of *Limnaea* and *Planorbis* were coloured with sudan black without previous bleaching, it could be seen plainly that the globules were much darker but the batonnettes were not; and when cells were bleached entirely and then coloured with sudan black, the globules reappeared in exactly their former sizes and positions but the batonnettes did not. It appears, therefore, that these globules, stained by osmium tetroxide, are the same as those produced by Thomas using sudan black after bleaching. Osmium-impregnation appears to be capricious. Either the batonnettes alone, or both batonnettes and globules may be stained. Thomas's variant (p. 455) of the Mann–Kopsch technique was tried on *Planorbis* neurones. Only globules were seen, except for a very faint indication of batonnettes in a very few cells. These globules agreed in all respects with those seen by the Mann–Kopsch technique.

With *Helix* material fixed in Helly's fluid, postchromed, stained with acid fuchsine as for mitochondria, and differentiated with sodium carbonate solution (Cain, 1948), no mitochondria were seen, but spheroid complexes with very fuchsinophil rims and colourless interna were present and agreed entirely with Thomas's description (p. 456).

With Baker's acid haematein test the picture obtained varied. The pyridine-extraction control always gave a completely negative result. The acid haematein test showed the cytoplasm either a clear yellow-brown with



Text-fig. 4. Camera lucida drawing of an acid-haematein preparation of *Helix* neurones. *c*, cell with the cytoplasm staining heavily throughout.

certain blue-stained bodies or intense blue-black throughout. In the latter case the cells were often slightly shrunken, and on occasion were confined to one part of the ganglion, within which every cell was blue-black. It is considered that such cells were damaged in some way and a lipophanerosis had taken place so that phospholipines were liberated in the cytoplasm. In the cortex of rat adrenals very similar appearances have been seen in those cells of the zona reticularis lying next to the medulla; this is a region in which necrotic cells are found. In this case, the liberation of phospholipine is a sign of the death of the cell.

In neurones not blue-black throughout, coloured spheroids are occasionally visible, again tending to be concentrated in the axon hillock (Text-fig. 4). In addition there are small coloured bodies, scattered in the cytoplasm, which appear to be the caps or rims associated with the smallest spheroid complexes in the living cell, the remainder of each complex being invisible in acid-haematein preparations. Some of the larger spheroids appear to have less coloured interna, but, as Thomas remarks (p. 452), this is not obvious, and

cannot be asserted with great conviction. Clouds of phospholipine occurred in some cells.

Material fixed for 3 days in formal-calcium was cut on the freezing microtome and the sections coloured with sudan black B. This method (Baker, 1944) showed large numbers of globules in the axon hillock, and others scattered throughout the cell. In every case it was the rim or attached granule that coloured with the sudan black. The picture was almost exactly that of living cells exposed to osmium tetroxide vapour. In all 3 species the cytoplasm coloured heavily throughout, and it was necessary first to remove this general colouring which indicates the presence of lipoids throughout the cytoplasm.

The yellow or orange-yellow pigment in the cells rapidly becomes colourless under the influence of light and air; the application of concentrated sulphuric acid produces a fine blue-green colour immediately. This had been noted by Smallwood and Rogers (1908), who concluded that the pigment was 'lipochrome'. Iodine in potassium iodide solution gives a deep violet. These facts indicate that the pigment is carotenoid, and this is confirmed by the blue colour given with a solution of antimony trichloride in chloroform. This reagent gives a blue colour with both vitamin A and carotene, and in spite of statements to the contrary cannot be used to distinguish between them unless heat is used (Andersen and Levine, 1935). As the chloroform tends to dissolve out the carotenoid, and heating accelerates this, it was found impossible to distinguish any colour on heating. The white precipitate of oxychlorides formed by antimony trichloride in contact with water tends to obscure the preparation.

In cells fixed for 6 hours in formal-calcium the carotenoid-containing granules take up far less sudan black than would fat-droplets of the same size. Lison (1936) notes that pure carotenoids are negative to lipoid reagents. This suggests that the largest granules are composed of carotenoids only among the lipoids, although the presence of proteins cannot be excluded. The granules in *Planorbis* and *Limnaea* often seem much more angular than those in *Helix* and give an impression of solidity.

It appears then that the complexes contain phospholipine and perhaps other lipoids in the rims, caps, or associated granules, and carotenoid in the interna. Vitamin A being much paler in colour than carotene, and giving the same reactions, cannot be excluded by the results given above, nor is its presence established. As there is no evidence for the manufacture of carotenoid by the Golgi apparatus as against its collection from other regions, it seems best to refer to its *accumulation* therein.

Discussion

The results given above agree with those of Thomas. It seems reasonable to conclude, as he does, that the filaments stained with Janus green appear in Mann–Kopsch preparations as the batonnettes and are the mitochondria. The spheroid-complexes are the Golgi bodies with their products. There is

a coincidence of structure and distribution-patterns of the globules (spheroid-complexes) seen in life and after treatment with all the following: neutral red, nile blue, methylene blue, acid haematein, Helly and acid fuchsine, formal-calcium and sudan black, and the Mann–Kopsch technique and Thomas's variant of it. This demonstrates clearly that they are all the same bodies. From the Mann–Kopsch preparations and the living cells stained with Janus green B it is clear that they are not associated with the mitochondria. Their identification with the Golgi apparatus is discussed by Thomas (p. 456). In structure they agree very well with bodies in other cells which are undoubtedly the Golgi apparatus (Worley, 1943, 1944, and 1946; Worley and Worley, 1943; Baker, 1944; Cain, 1947*a*).

The principal arguments that might be brought against this identification are that such bodies are not shown by the standard Golgi methods, that the batonnettes with their archoplasm are the Golgi apparatus, or that the use of neutral red chloride and other supravital stains cause the production of artifacts. That in these neurones the spheroid complexes are not shown by the standard methods is a much more serious objection. The Mann–Kopsch method does show it sometimes, less readily in *Helix* than in *Limnaea* and *Planorbis*, but it is quite easy to find cells in which nothing but the batonnettes can be seen. But the standard methods are wholly empirical, and, as far as is known, no Golgi apparatus is under obligation to appear when they are used. So very little is known about conditions at the inner surfaces of the cell and the conditions under which silver and osmium precipitates form. What is known about monomolecular layers on water-surfaces indicates that their behaviour can change very greatly with small changes in pH and the concentration of ions in the water (see e.g. Langmuir, 1934) and it would not be at all surprising if structures containing layers of oriented molecules should be very similar in morphology but very diverse in their behaviour, differing, particularly, in different sorts of cell, and most divergent in the most specialized. The fact that these bodies do not usually appear with the standard Golgi methods does not rule out the possibility that they are the Golgi apparatus. The objection to the use of neutral red and similar stains can hardly be upheld in this case because it is not a question of making structures visible, but of staining structures already visible in the cell.

The view that the batonnettes are the Golgi apparatus has been very well supported. This was based on their regular appearance with the standard Golgi methods, and a supposed homology with the lepidosomes of the pulmonate primary spermatocyte. But there seems to be no special reason for carefully selecting cells which show batonnettes only and excluding one showing the spheroids as well; and the batonnettes do correspond with the mitochondria as shown by Janus green, and do not correspond with the spheroid complexes seen in the living cell and ignored by upholders of the batonnette theory. In *Limnaea* and *Planorbis* the batonnettes are less readily shown by the Mann–Kopsch method than in *Helix*, and the spheroids perhaps more readily. Perhaps if investigations on gastropod neurones had started

with *Planorbis* instead of *Helix*, as much attention might have been given to the spheroids as to the batonnettes.

Summary

Repetition of some of Thomas's (1948) work on *Helix* neurones and its extension to neurones of *Planorbis* and *Limnaea* confirms his conclusions that the batonnettes shown by standard Golgi methods are mitochondria, and the Golgi apparatus is represented by spheroid complexes, scattered throughout the cell but tending to be concentrated in the axon hillock.

The spheroid complexes appear to consist of an externum, continuous or not, which contains phospholipine and possibly other lipoids, and an internum in which carotenoids are accumulated. This accumulation is greater in *Limnaea* and *Planorbis* than in *Helix*.

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A Further Note on Nile Blue

BY

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IN a previous paper (Cain, 1947) it was shown that nile blue can be used to distinguish between acidic and non-acidic lipoids, and that lecithin stained very deep blue with a 1 per cent. solution, while oleic acid was only faintly coloured. Since lecithin stains, it was suggested that other lipines might stain blue also and that the oleic acid used might be impure.

A sample of phosphorus-free oleic acid was dried over anhydrous sodium sulphate for three months. One portion was then shaken with 1 per cent. nile blue aqueous solution, and solid nile blue was added to another. The acid in contact with the solution coloured blue immediately. The other portion showed only traces of blue (appearing red by transmitted light because of fluorescence) after 2 hours. After 12 hours it had darkened considerably, but did not reach equality with the first portion for 24 hours. Since the dried acid takes up nile blue so much more slowly than that in contact with water, it appears that the blue-staining of oleic acid is connected with its power of imbibing water, and as it is highly unlikely that dry oleic acid will be met with in animal tissues, no use can be made of blue-staining with 1 per cent. nile blue to differentiate between lipines and fatty acids.

A sample of pure galactolipine (phrenosin and kerasin) which gave a negative reaction with the acid haematein test was found to stain deep blue with 1 per cent. nile blue. The substance was attached to a coverslip by gentle warming and then cooling. Kaufmann and Lehmann (1926) obtained a negative result with pure phrenosin and with pure kerasin, probably because, as with lecithin, their method of impregnating pith was not suitable. Nile blue, therefore, cannot be used to distinguish between various members of the lipines.

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Ester Wax as a Medium for Embedding Tissue for the Histological Demonstration of Glycogen

BY

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Introduction

IT is generally recognized that glycogen is a very labile substance which disappears rapidly from tissues unless treated by special methods of fixation and embedding. The recognized and widely used techniques, advocated by the standard microtomical treatises, call for fixation in a glycogen-precipitating fixative (absolute alcohol; picro-formol-alcohol; picro-dioxane) followed by celloidin embedding.

Since glycogen is considered to be readily soluble in water, if paraffin sections are to be used, special precautions are advocated: sections must be flattened on slides with 70 per cent. alcohol instead of albumen-water; mounted sections, after removal of wax, must be brought into absolute alcohol and then into 1 per cent. celloidin in absolute ether. This latter process covers the section with a thin film of celloidin which 'seems to prevent the diffusion of the glycogen from the section into the water' (Carleton, 1938). Most workers further emphasize that water must be avoided during the subsequent staining processes. On the other hand, Bensley (1939) in a recent account of staining methods for glycogen makes no mention of the necessity for the celloidin film, but merely states that 'tissues may be embedded in paraffin or celloidin'; and when staining 'sections may be brought down to water'.

In recent work on cestode physiology we have had available a considerable quantity of material rich in glycogen, namely, the plerocercoid larvae of the cestode *Ligula intestinalis* which contain seldom less than 30 per cent. glycogen (dry weight; Markov, 1939). It has thus been possible to test the embedding methods on this material on an extensive scale. Confirmatory experiments were carried out with rabbit liver.

Experimental Results

Ligula intestinalis

Paraffin wax. Pieces of fresh larvae removed from a fish immediately after pithing were fixed in hot (60° C.) picro-formol-alcohol (absolute alcohol saturated with picric acid—90 c.c.; neutral formol—10 c.c.), embedded in paraffin wax, sectioned at 5μ, and stained with iodine or Best's

carmine using the modifications of Bensley (1939). Results were briefly as follows:

(i) Some blocks gave sections rich in glycogen, whereas others gave sections poor in glycogen.

(ii) Blocks which gave sections poor in glycogen, and which had only been sectioned about half-way through, when re-embedded in pure wax for long periods (at least overnight) now gave sections rich in glycogen!

(iii) When a 'short-embedded' block, partly sectioned, was immersed in aqueous iodine, the *centre* of the tissue at the block face stained immediately, whereas the periphery stained only after some minutes' immersion.

(iv) Sections from 'long-embedded' blocks or re-embedded blocks, provided they cut properly (which they rarely did), were as rich in glycogen if floated on albumen-water as on 70 per cent. alcohol.

Ester wax. Prolonged embedding in paraffin wax had the effect of making material very refractive and sections were usually crumbly and difficult to cut. This latter difficulty was overcome satisfactorily by using the very hard ester wax recently introduced by Steedman (1947). The procedure adopted was as follows:

Fix in picro-formol-alcohol: 2 hrs.
Absolute alcohol (3 changes): 12–24 hrs.
Absolute alcohol/ester wax (1:1): 2 hrs.
Pure ester wax: 3 hrs.
Pure ester wax: 12 hrs.—overnight.

Sections were cut with ease at 5μ, flattened on albumen-water in the usual way, and stained as before. All sections from blocks embedded in this manner contained great quantities of glycogen filling every available inter-cellular space in the cestode tissue.

In order to test the lability of glycogen, a number of sections were brought down to water, left standing in water, and removed and stained at intervals of one day to determine how rapidly glycogen disappeared from the sections. In contrast to expectations, it was found that *such sections could remain in water for periods of 5–7 days without any appreciable glycogen loss.*

Liver

The results outlined above—though agreeing with those obtained from similar but much-less-detailed experiments on larval *Diphyllobothrium* (Smyth, 1947)—were so contrary to the accepted views on the lability of glycogen, that it was considered possible that glycogen in such cestode tissue might exist in a form different from that in mammalian tissue, although results of chemical analysis suggests that glycogen in cestodes does not differ significantly from mammalian glycogen (Brand and Oesterlin, 1933; Wardle, 1937; Salisbury and Anderson, 1939).

In view of this possibility, a confirmatory series of experiments was therefore arried out using as a test material pieces of liver from a rabbit previously fed n carrots for 3 days—following the usual practice. Pieces 2–3 mm. thick ere fixed in picro-formol-alcohol and embedded in (*a*) paraffin wax, using ormal embedding times, i.e. 'short embedding'; (*b*) paraffin wax, using vernight embedding, i.e. 'long embedding'; (*c*) ester wax.

It was found that there was no significant difference between (*a*) and (*b*), ut that on the whole sections from these two series were somewhat inferior to ster wax sections as regards the discreteness of the globules and the brightness f the staining. All series of sections could remain standing in water for very onsiderable periods without glycogen loss. Paraffin wax sections showed ome loss after 5 days, but ester wax sections showed not the slightest trace f loss even after 7 days' immersion in water, and it was impossible to dis-inguish sections that had been soaked in water for this period from those hich had been stained without immersion.

Discussion

The main results in these experiments that require explanation are: (*a*) ieces of *Ligula* require 'long embedding' in paraffin wax in order to retain lycogen, whereas 'short embedding' is sufficient for thin pieces of liver; (*b*) ster wax sections are superior to paraffin wax sections; (*c*) sections of properly mbedded material when brought down to water can remain in water for long eriods without appreciable glycogen loss.

(*a*) The fact that by prolonged embedding of *Ligula* in paraffin wax the lycogen is held, whereas by short embedding it is not, suggests that with hort embedding the wax molecules do not completely permeate into the reat amorphous masses of glycogen present in the inter-cellular spaces. This iew is substantiated by the fact that when a short-embedded block, partly ectioned, is immersed in iodine the tissue stains instantly in the middle egion yet only slowly at the periphery. This we interpret as indicating that he wax, which slows up the movement inwards of the aqueous iodine, has ever reached the centre. If the glycogen in such a block was not firmly held y the wax, on sectioning some would fall out as a powder and other masses ecome loosened to such an extent that they could be lost by mechanical neans in later mounting and flattening. This is exactly what happens to ections cut from the block just mentioned; although the face of the block fter standing in iodine takes up the iodine intensely—thus showing glycogen o be present—yet sections from this block when stained contain little lycogen in the middle region, i.e. the region where the wax had not properly ermeated. If such a block be re-embedded in wax for a long period, it gives ections uniformly rich in glycogen.

It thus seems reasonable to conclude that the glycogen is lost in cutting ections and in the subsequent flattening processes. This must be considered o be purely a *mechanical* effect due to the fact that glycogen is not completely ermeated in wax in short-embedded blocks and is thus only loosely held.

If this hypothesis be true, the question immediately arises—why is short embedding sufficient for pieces of liver? This result can be accounted for when the amount of glycogen in liver cells is compared with that present in *Ligula*. In the former, glycogen is present only as small globules which can be penetrated on all sides by wax, whereas in the latter enormous masses of glycogen fill the parenchymal and muscular inter-cellular spaces. It seems self-evident that the time required to permeate the dense masses in *Ligula* will be considerably longer than that required to permeate the small globules in liver cells.

Previous workers have drawn attention to the flattening procedure as being a critical one, as the aqueous albumen would seem a likely place for the generally supposed highly labile glycogen to diffuse out. In one detailed experiment 10 sections of liver were mounted on water, and the same number on 70 per cent. alcohol; it was impossible to distinguish any difference in the amount of glycogen in the slides of the two series. Since this original experiment we have mounted many hundreds of sections flattened on albumen-water without ever getting results suggestive of a loss at this stage, provided material was properly embedded.

(*b*) If good results depend only on getting the paraffin wax right into the glycogen, it is to be expected that long-embedded paraffin wax blocks should give as good results as ester wax. The reason why ester wax gives better results we have already indicated, i.e. prolonged embedding makes tissues exceedingly hard and refractive with the result that sections are frequently difficult to cut and often crumbly, torn, or wrinkled, thus giving glycogen a chance to be lost at a later stage. Ester wax, due to its celloidin-like toughness and strength, permits sectioning of very hard material with great ease and does not introduce the difficulties resultant of paraffin wax embedding. We do not consider, therefore, that *per se* ester wax has any intrinsic property for retaining glycogen, but that like celloidin it merely overcomes, due to its toughness, the technical difficulties introduced by alternative methods.

(*c*) It has long been accepted that glycogen in sections is a highly labile substance, and for that reason most previous techniques have emphasized that sections should be handled without taking them below 60 per cent. alcohol—the strength at which alcohol precipitates glycogen from aqueous solutions. When, for example, aqueous reagents—or reagents with a low alcohol content—were used in the staining technique (i.e. as in counterstaining in the Best carmine method), the celloidin film technique was introduced to prevent diffusion of the labile molecules into the surrounding medium.

Basing our hypothesis on the above results, we believe that—providing material has been properly fixed, thoroughly dehydrated, cleared and embedded, sections not damaged in cutting, and smoothly flattened—when wax is removed and slides brought down to water, no glycogen is lost by dissolving in water. The fact that slides can be stored in water for 7 days and still be packed with glycogen must force us to revise prevalent ideas concerning the

lability of glycogen, and points to the inevitable conclusion that glycogen is either in an insoluble form at this stage or is 'held' in some way.

It is at once apparent that the answer to this problem lies in a greater knowledge of the structure and properties of glycogen. Recent work has shown that glycogen is a mixture of two complex polymers with highly branched molecules: *lyo*-glycogen, whose molecule is weakly linked to proteins and is *soluble*, and *desmo*-glycogen, whose molecule is strongly linked to proteins and is *insoluble* (Myer, 1942; Carter and Record, 1939). Since liver-glycogen contains only about 15 per cent. of the *desmo-* form (Genkin, 1946) it cannot possibly account for the amount present in our sections; we resort therefore to the alternative conclusion that the *lyo-* form has at least been partly—if not entirely—retained. We believe that the *lyo-* form does not diffuse from sections immersed in water because its large branched molecules are held by the associated protein network which has been precipitated around them. Lison (1936) has already put forward this hypothesis in more general terms, and, as he has pointed out, it adequately explains why a protein-coagulating fixative such as picric acid—while not precipitating glycogen *in vitro*—is an excellent fixative for glycogen in tissues.

Summary

1. The effect of paraffin wax embedding on the lability of glycogen in sections has been tested using as material (*a*) plerocercoid larvae of *Ligula intestinalis*; (*b*) rabbit liver.

2. It was found that glycogen in tissues was very impermeable to wax, and where large masses of glycogen occur prolonged embedding is essential.

3. Improperly embedded material lost glycogen easily; it was concluded that this loss takes place during the processes of cutting and flattening sections.

4. The effect of prolonged embedding was to make tissue hard and refractive. This difficulty was overcome by embedding in Steedman's ester wax.

5. Ester wax blocks allowed thin sections of very hard material to be cut with ease.

6. Ester wax sections brought down to water did not lose glycogen even after standing in water for 7 days.

7. It is emphasized that glycogen exists in two forms: an insoluble *desmo-* form and a soluble *lyo-* form. It is suggested that the latter does not dissolve from sections in water because its highly branched molecule is held by the coagulated protein network.

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A Simple Method for Orientating Small Objects for Sectioning, with Special Regard to Nematodes

BY

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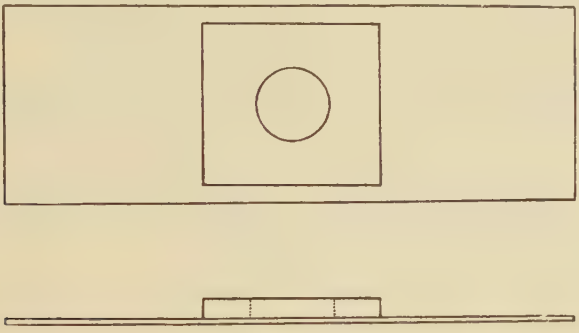
With one Text-figure

FOR solving certain anatomical problems in free-living nematodes serial sections are necessary. Various methods of orientation proved unsatisfactory for these small objects (in this case 700μ in length and 30μ in width). The following method was worked out and found excellent:

In a drop of water the nematode is heat-paralysed, stretched at 50–60° C., and pipetted into Bouin's mixture (Graupner). After 24 hours it is transferred to 30 per cent. alcohol (4 hours), 50 per cent. alcohol (3 hours), a mixture of 50 per cent. alcohol and aniline (1 : 3) (1 hour), pure aniline (1 hour), bergamot oil (3 hours), paraffin wax I (3 hours), paraffin wax II (1 hour), in which it is embedded.



TEXT-FIG. 1

All the manipulations except the last one are performed in the usual way and practically no shrinkage takes place.

The difficulties in orientation preceding the embedding are overcome in the following way:

A square glass plate 2 mm. thick and with side of 22 mm., provided with a central circular hole of diameter 15 mm., is fixed by means of Canada balsam to a microscope-slide (see Text-fig. 1). When the Canada balsam has dried the hole is filled with the paraffin wax II, into which the object is transferred from paraffin wax I for orientation and embedding. By means of a heated needle the object is placed 1 mm. above the bottom in the paraffin

wax, which as far as possible is kept in a liquid state by cautious heating over a small flame. These manipulations are performed under a binocular microscope at about 40-fold magnification. Under these circumstances it is easy to orientate the object in such a way that the intended plane of section is exactly parallel with the bottom. When the object has been placed in position the slide is immediately plunged into water of 10–12° C. (If the water has a higher temperature, the needles of solid paraffin will have the same order of size as the object, and a very loose and unsatisfactory embedding results.)

When the paraffin wax has become solid a block with sides about 5 mm. long is cut out enclosing the object.

A block of wood with a block of paraffin wax fused on it is placed in the microtome and sections are cut until a convenient area of its surface is plane.

By means of a forceps the object block with the plane lower surface downwards is held 5 mm. above the block in the microtome. A very hot lancet is placed in the interspace between the two blocks (without touching either of them). When the radiant heat has melted a fine film of paraffin wax on the two surfaces, the blocks are rapidly fused to each other. After cooling, the block-holder of the microtome is lowered 2 mm. by means of the screw and sectioning may take place. If the radiant heat has been applied accurately, the plane of section will be quite parallel to the plane lower surface of the block containing the object.

Thus an exact orientation (at magnification $\times 40$) is obtained and maintained during sectioning in a purely mechanical way without the drawbacks of a subjective estimation, which is practically impossible in the case of these small objects even if they have been stained beforehand.

By employing this method it has been possible to obtain exactly longitudinal sections in a large number of these small objects, which cannot be seen with the naked eye.

Exactly the same technique has been satisfactorily employed for the sectioning of Protura and Mallophaga—without the use of diaphanol.

A New Single-control Micromanipulator

BY

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AND

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With one Plate

Introduction

SEVERAL types of micromanipulator have been described (for reviews see Peterfi, 1928; Chambers and Kopac, 1937; Seifriz, 1936). The growing importance of micrurgical techniques in bacteriology, electrophysiology, embryology, plant physiology, and experimental cytology have focused attention on the problem of micromanipulator design. The question has assumed a new importance with the recent development of two revolutionary advances in microscopy. The first of these is the high-performance reflecting microscope of Dr. C. R. Burch (see Burch, 1947; Barer, 1948*a*). The instrument in use at Oxford has a working distance of 13 mm. when used at N.A. 0·65. This long working distance is exceptionally useful for microdissection, as it enables the usual type of moist chamber and hanging drop preparation to be dispensed with. It is now also possible to perform micromanipulation on the surfaces of intact organs *in situ*, e.g. the liver, spleen, kidney, or brain of anaesthetized animals (Barer, unpublished results). The second outstanding advance in microscopy has been Zernike's phase-contrast method (Zernike, 1942; Burch and Stock, 1942; Barer, 1947*b*, 1948*b*). This enables living unstained cells to be studied under optical conditions vastly superior to those hitherto available. There is no doubt that much early work will now have to be repeated and extended by the use of this method. The technique of microdissection by phase-contrast illumination is far from easy, but the results amply repay the trouble.

Features Desirable in a Micromanipulator

1. *Single Control*

Most micromanipulators, e.g. those of Chambers and Peterfi, depend on three main controls, one for each direction in space. These usually take the form of screws which operate rack-and-pinion mechanisms or cause the spreading-apart or sliding motion of metal plates. While such methods of control may give very precise linear movements, the latter are rather limited

and unnatural, since in order to go from point A to point B, one can only travel via X, Y, and Z. This would not be a very convenient way of writing one's name, nor is it the ideal method for microdissection. The ideal micromanipulator should enable the operator to move an instrument smoothly and rapidly along any desired path between two points in space. So far as we are aware this ideal condition has yet to be achieved, and the mechanical problems involved are very formidable. Fortunately an approach can be made to the problem if we are prepared to sacrifice complete smoothness of control in three dimensions, substituting instead smooth movement in one plane combined with an independent movement in a direction perpendicular to this plane. Simple micromanipulators based on this principle have been described by Buchthal and Persson (1936) and by Schuster (Barer, 1947*a*). These instruments allow any desired movement in a vertico-lateral plane, with a screw control for antero-posterior movements. Their great advantage is that all movements are operated from a single control, which remains in the hand during the entire operation, but their inherent weakness lies in the fact that for most high-power work the greatest precision of movement is required in the *horizontal* plane, i.e. the plane in focus under the microscope. Thus, although such micromanipulators are quite satisfactory for use at medium magnifications (up to about 500 times), their use at higher powers demands considerable skill. Of the instruments which allow complete freedom of movement in a horizontal plane perhaps the best known is that of de Fonbrune (1932, 1937). This outstanding high-precision design has been developed over a period of many years and the present model must rank as one of the finest available. It is unusual in that it works on pneumatic principles. Air pressure from three mutually perpendicular pistons is transmitted through three rubber tubes, on to three tambours, resembling aneroid barometers, which are connected to a lever holding the micro-instrument. The pressure in the pistons is controlled by a single handle. Movement of the handle in a horizontal plane moves one or both of the horizontal pistons, and vertical movement is obtained by a screwing motion of the handle. With practice something very near to complete freedom of movement in three dimensions can be achieved. The de Fonbrune micromanipulator incorporates a number of other important features which will be referred to below.

2. *Freedom from Vibration*

The elimination of vibration is essential in micrurgy. Factors which may help in this respect are (1) massive construction; (2) clamping the micromanipulator and microscope to a common base-plate; (3) remote control. Most commercial micromanipulators rely on factors (1) and (2), either alone or together. Massive construction is usually no disadvantage in high-power cytological work, but it may be an encumbrance if the instrument is to be used for other types of work, where it may require to be poised in mid-air at an angle. The same may sometimes be said of factor (2). Some micromanipulators are only designed for use for one specific purpose and a more

or less fixed base-plate assembly is provided. This greatly limits the versatility of the instrument and in general it is preferable to have an instrument which can be made quite independent of the microscope if required. A common base-plate with clamps can always be added as an accessory. The Schuster micromanipulator is unusually adaptable in this respect. Although usually mounted on a heavy base, the effective part of the instrument can be detached and mounted in any position or orientation on a clamp or sliding bar. This makes it particularly useful for accurate positioning of electrodes. Remote control, i.e. absence of rigid connexion between the control screws or handle and the micro-instrument holder itself, is obviously a valuable feature in reducing vibration, and accidental jarring of the controls may not be transmitted to the micro-instrument itself. Although remote control was introduced into a form of the Chambers micromanipulator, the de Fonbrune is the remote-control instrument *par excellence*. Here the single-control handle and the instrument proper are built as two independent units, connected only by a considerable length of flexible rubber tubing. The instrument holder is comparatively light and delicate but freedom from vibration and accidental jolts is assured by this independence, which also enables the instrument to be used to some extent for other than cytological work. For maximum stability and robustness there is no doubt that massive construction is an advantage. On the other hand, remote control is often very convenient for ease of manipulation and may enable a lighter type of construction to be adopted.

3. *Freedom from Play* (*backlash or lost motion*)

It is essential that the instrument holder should respond without delay to any movement of the controls, and that there should not be any further movement or 'creep' on sudden removal of the hand. This 'dead-beat' condition can only be achieved by careful attention to details of design. If differential screw feeds or rack-and-pinion movements are used they must be made with a considerable degree of precision. Sliding surfaces and bearing points must be of suitable material and should be so designed that the effects of wear are eliminated. Multiple levers and cam mechanisms are on the whole to be avoided as they are rarely free from play. This fault makes the design of micromanipulators on the usual principles of a pantograph rather impracticable.

4. *Variable Sensitivity of Control*

It is extremely useful to be able to vary the sensitivity of control according to the magnification of the microscope. If this cannot be done it may be found that work under low powers is too slow, especially with screw controls, as it may take a long time to traverse the field of a low-power objective. Again with screw controls a large field can as a rule only be traversed by a series of turns of the screw, thus leading to intermittence and jerkiness of operation. Some degree of variation of sensitivity can be introduced into the Chambers and rack-and-pinion instruments by attaching special levers on to

the operating screws, but this profusion of rods jutting out in various directions of space is clumsy and inconvenient. One of the most successful methods of achieving smooth and *continuous* variability of sensitivity is that adopted by de Fonbrune. A sliding collar is fitted around the vertical control handle. Rods run from this collar to each of the two pistons which control movements in a horizontal plane. A movement of the sliding collar alters the angle of movement of these piston levers and thus the range and sensitivity of motion in the horizontal plane. The vertical movement is unaffected, but variation of this is relatively unimportant for most work. This mechanism is exceedingly valuable, but three disadvantages should be noted. In the first place the transition from rather coarse to very fine movement is not linear but tends to be rather abrupt—most of the reduction takes place in the final few millimetres of travel of the sliding collar. This is not a very serious fault since the variation is continuous. Secondly, the range of movement diminishes with increasing sensitivity; for example, if the sensitivity is doubled the range of movement is halved. This is inconvenient if very fine work has to be done over a very large field, although this requirement is rare in practice. In principle a screw mechanism could be made free from this fault. The third disadvantage is rather more serious. A movement of the sliding collar not only alters the angle of movement of the horizontal piston levers but moves the pistons bodily by a small amount, thus shifting the micro-instrument. The latter thus has to be recentred and may even move out of the field of view altogether.

5. *Limitation of Movement to the Field of View*

In most micromanipulators no provision is made for this. Thus if by chance the micro-instrument should wander out of the field of view it may be very difficult to find it again. This is particularly so when working at high magnifications, when the only practicable plan may be to return to an objective of lower power. In the de Fonbrune instrument the movement of the control handle is limited by a metal circle. The range of movement can be made to correspond exactly with the field of view by adjustment of the sliding collar mentioned above.

6. *Rapid Centration*

Some form of coarse movement to enable rapid centration of the micro-instrument in the field of view is highly desirable. This may not be so important in instruments made to clamp in a fixed position relative to the microscope, and when micro-instruments of a standard length are used, but it is almost essential in other cases. A coarse vertical motion is also desirable.

7. *Robustness, Price, &c.*

This is to some extent connected with the question of massive construction. In choosing between two instruments of roughly equal performance it is natural to decide in favour of the more robust and less easily damaged. It is

also important that any accidental damage should be capable of easy repair, preferably by any competent workshop technician, and that spare parts should be available at low cost. This puts delicate instruments and those involving high-class precision engineering at a disadvantage. It is often possible to cut down the price of an instrument very considerably by employing methods of design not involving high-precision fits. Price and robustness are important considerations in a micromanipulator, since for most work a pair of instruments is required.

The New Micromanipulator

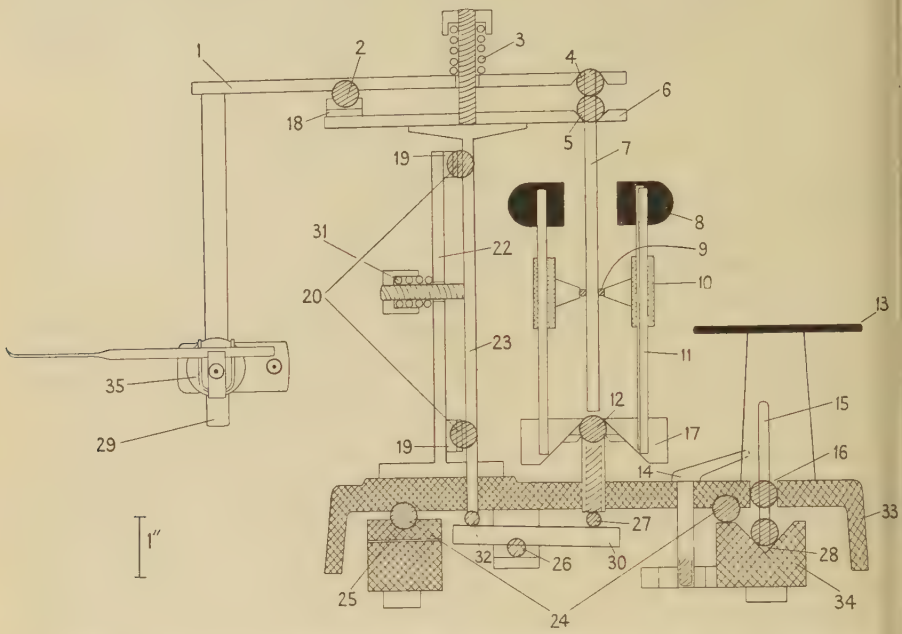
Although the resemblance is at first difficult to find, the present instrument began as a simple modification of the Schuster micromanipulator. As we have seen, the chief drawback of the latter is the fact that smoothest motion occurs in a vertical plane, whereas we should like it in the horizontal plane. A fairly obvious modification was thus to turn the instrument through 90°, with the control handle vertical, and to attach the micro-instrument holder to the upper sliding plane. The first rough models gave surprising results. The action in a horizontal plane was beautifully smooth and controllable, and by attaching the control handle to a heavy block of metal which could slide on a polished surface it was possible to trace most complicated patterns with extreme rapidity. Unfortunately, in order to obtain perfect uniplanar motion the vertical movement had been sacrificed. Experiments were made with a simple tilting vertical motion by means of a screw fixed through the base of the instrument, but this did not prove sufficiently good for high-power work. At the same time the first results seemed so promising that it was felt justifiable to build a much more ambitious model incorporating several of the desirable features described above.

The construction of the latest version of the new instrument will be seen from the diagram (Text-fig. 1) and photograph (Pl. I). The steel ball-bearings (2) of the horizontal movable brass plate (1) rest in a groove and a flat (18) on the horizontal fixed brass plate (6), clamped by the adjustable spring (3). The double-ball-ended lever (4, 5, 7) locates in two cones in the plates (1) and (6) and is gripped by three small steel balls (9) mounted on a slider (10) which moves on three rods (11) fixed to the plastic handle (8) and the coned base (17). Various types of bearing points, including jewel tips, were tried at (9), but the steel balls were found to be most satisfactory and did not score the lever (7).

The coned base (17) is clamped to the screw-mounted steel ball (12), the lower end of the screw making contact with a steel ball (27) fixed to the lever (30), which moves about the ball fulcrum (26). The balls (20, 20), fixed to the vertical movable plate (23), rest in V-grooves and a flat (19, 19) of the vertical fixed plate (22). These two plates are held together by the adjustable spring (31). The lower end of the vertical plate (23) is fixed to a ball (32) resting on the lever (30). The balls (24, 24) of the heavy base (33) rest in grooves (25) and flats on the undercarriage (34). The base (33) and undercarriage (34)

can be fixed together by the clamp (14). The short double-ball-ended lever (15) locates in a hole (16) in the base (33) and in a cone (28) in the undercarriage (34).

The micro-instrument holder (35) is clamped to the tool post (29) and is adjustable vertically, horizontally, and angularly before locking.



Text-fig. 1. Diagram of micromanipulator. For explanation of the numbers see text.

Operation

The micromanipulator is placed at a convenient distance from the microscope, and if desired it can be clamped to a common base. (The three supports on the undercarriage can be conveniently sunk in three slots.) The micro-instrument holder is adjusted to a suitable height and the micro-instrument inserted. It should be noted that the type of holder we have found best is a small version of the Singer clamp (Registered Design No. 847073). This holder will accommodate anything from a very fine needle to a tube 9 mm. in diameter, thus allowing the use of non-polarizable micro-electrodes of wide bore. The micro-instrument is held in place by pressure from a metal leaf against two V slots. The micro-instrument does not have to be held horizontally as is the case with many other types of holders, but can be used at any desired angle. The desirability of having a rack-and-pinion movement of the holder on the post (29) was considered, but it was felt to be an unnecessary refinement for most work.

The clamp (14) is now loosened and the tip of the micro-instrument is centred rapidly in the field of view by means of the handle (15) which causes the base (33) to slide on the undercarriage (34). This movement of the base

on the undercarriage is of wide range and can even be used for very low-power micromanipulation. As soon as the micro-instrument is centred the clamp (14) is tightened, preventing any further movement of the base.

The hand of the operator rests on the plastic hand-rest (13). The fingers grasp the handle (8). Movement of the handle produces reduced motion through the balls (9) working on the ball (12) to the lever (7). This moves the plate (1) to which the tool post (29) is fixed. The plate (1) can only move in a horizontal plane. Thus a rocking movement of the handle (8) enables any desired curve to be traced in a horizontal plane, at any desired speed. Twisting of the handle (8) rotates the screw-mounted ball (12). This moves the lever (30) about its ball fulcrum (26), and allows the plate (23) to be raised or lowered against the fixed plate (22). The two horizontal plates (1) and (6) and with them the tool post (29) are thus moved vertically.

The slider (10) enables the sensitivity of the instrument to be varied through a wide range. When at its highest position, close to the handle (8), a fairly coarse movement of wide range is obtained, suitable for low-power work. In the lowest position, close to the coned base (17), the movement is very delicate, but reduced in range. At the same time it will be noted that the movement of the handle is automatically limited by its circumference coming into contact with the lever (7). This restricts the range of movement of the micro-instrument tip. In practice it will be found most convenient to move the slider (10) until a position is found at which the range of movement of the micro-instrument tip corresponds with, and is limited to, the field of view of the microscope in use at the time. The sensitivity of the instrument can be altered at will without any undesirable movement of the micro-instrument.

Remarks

We are now in a position to consider how far the present instrument meets the requirements (1) to (7) discussed above.

1. Although we have not achieved perfect control in three dimensions we have virtually perfect control in the two dimensions of a horizontal plane, combined with vertical movement controlled by the *same single handle*. The majority of cytological work can be carried out in a horizontal plane with only occasional use of the vertical movement. One minor fault must be pointed out here. It would be desirable to be able to carry out a vertical movement without risk of a slight horizontal displacement. This is sensibly so at low and medium sensitivities. At very high sensitivities, however, when the slide (10) is close to the coned base (17), the effective leverage is so great that the horizontal movements are carried out against appreciably less resistance, making it more difficult to twist the handle (8) without producing some slight horizontal displacement, though this can be done with practice. However, this tendency can be reduced to a large extent by tightening the screw spring (3), which increases the resistance to horizontal movement. The tension of the same spring can be adjusted for maximum ease of manipulation at any sensitivity.

2. The instrument will be found to be remarkably free from vibration. Even if the handle (8) is struck against the lever (7) with considerable force the vibration of the micro-instrument is relatively slight and rapidly damped. Movements of the hand on the hand-rest are usually without obvious effect. This feature has been achieved largely by massive construction.

3. Despite numerous trials no appreciable play has been noticed. This very important result has been achieved entirely without any fine-limit precision-engineering methods, but mainly by attention to the design of the bearing surfaces. Any tendency to the development of play as a result of wear can be taken up by adjustment of the tensions of the two springs (3) and (31). The design is such that the bearing surfaces would merely 'bed' into one another as wear occurs.

4. Sensitivity can be varied quickly and easily over a wide range, and without moving the micro-instrument. The variability is continuous and sensibly linear, with no sudden transition from coarse to fine movement. It will be noted, however, that the range is automatically reduced as the sensitivity is increased.

5. The range of movement can be limited at will to the field of view.

6. Rapid centring is achieved by movement of the base (33) on the undercarriage (34).

7. The instrument is exceptionally robust. There are no delicate components whatsoever, and no high-precision work is involved. Should repairs ever be necessary they can be carried out by any competent workshop technician. It may perhaps be stated that the instrument has been subjected to considerable mechanical violence on a number of occasions in order to test its strength, and on no occasion has damage ensued. The same robustness is evident in the very versatile micro-instrument holder. The lack of any high-precision work enables the instrument to be made relatively cheaply, bringing a *pair* of micromanipulators within the reach of most investigators. The possibility that the instrument might be used at marine biological stations has been considered, in deciding the best type of corrosion-resisting finish. Corrosion of metals by sea-water is a very difficult problem, but it is felt that chromium plating combined with reasonable care offers the most practical solution.

In conclusion it may be mentioned that the instrument has been tried out for a number of purposes, including microdissection by phase-contrast illumination, and has proved very successful in every way.

We wish to thank all those, too numerous to mention, who have aided us by their discussion, criticism, and advice. In particular Mr. P. J. Peade and Mr. J. Parkinson have been most helpful. We also wish to place on record our indebtedness to certain principles of design laid down by the late Dr. W. N. Bond.

R. BARER AND A. E. SAUNDERS SINGER—PLATE I

SUMMARY

New developments in microscopy and electrophysiology have brought a renewal of interest in methods of micromanipulation.

A number of features desirable in the design of a micromanipulator is discussed.

A new micromanipulator is described. Its outstanding features are (1) single control; (2) massive construction with freedom from vibration; (3) freedom from play; (4) continuous variability of sensitivity; (5) limitation of range of movement to the field of view; (6) rapid low-power centring; (7) unusual robustness combined with delicacy of movement achieved without any high-precision methods. The performance of the instrument is adequate for high-power cytological work and for microdissection by phase-contrast illumination.

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SINGER MICROMANIPULATOR

(patent application 11559/48)

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